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COMPARATIVE CLINICAL AND BIOLOGICAL EFFECTS OF ALKYLATING AGENTS

BY

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COMPARATIVE CLINICAL AND BIOLOGICAL EFFECTS OF ALKYLATING AGENTS*

Consulting Editor
DAVID A. KARNOFSKY

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INTRODUCTORY REMARKS

By L. H. Schmidt

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In opening this monograph it seems appropriate to cite a series of observations made by Paul Ehrlich in 1898[1] in a manuscript dealing with the relationships between chemical constitution, distribution, and pharmacological action. In this report Ehrlich said:

"I have made extensive experiments with many hundreds of different compounds and in all of these I have discovered only one substance to which I am inclined to ascribe a substituting action on protoplasm. This substance, vinylamine, discovered by Gabriel, is formed by extracting bromine from bromoethylamine. . . . Marckwald has shown that this substance cannot, as was first supposed, contain a double bond. It can therefore only possess a constitution of dimethylenimine. In view of this a complete analogy exists between ethylenimine and ethylene oxide. . . . These two substances, ethylenimine and ethylene oxide, are highly toxic compounds, as has been shown by the research of Levaditi and myself. . . . The pathological changes excited by dimethylenimine are especially interesting. Administered to a great variety of animals in doses which caused death after one and a half or two days or more, this substance causes total necrosis of the renal papillae. In the rabbit Levaditi found besides this marked changes extending from the pelvis of the ureter to the urethra and consisting of necrosis of the lining epithelium, hemorrhages, and edema. Everyone who has learned to recognize these changes, absolutely unique in pathology, would be forced to the assumption that this localization is dependent on a direct attack of the vinylamine on the affected epithelium, the ethylenimine group entering the protoplasmic molecule."

These references to ethylenimine and ethylene oxide seem to represent the first recognition of the unique biological features of alkylating agents. It was to be more than forty-five years, however, before these observations were applied to the treatment of neoplastic diseases, and another three years before there was general knowledge of this application. It was not until 1942 that Goodman and Gilman, while working on the pharmacology of certain nitrogen mustard derivatives, noted the effects of these compounds on lymphoid tissue and rapidly dividing cells and suggested the possibility of their use in certain neoplasms of lymphoid character. This led to studies on experimental neoplasms and, subsequently, to clinical investigations at Yale University, New Haven, Conn., which showed that methyl-bis-(β-chloroethyl)-amine had significant activity against Hodgkin's disease and evoked limited regressive changes in certain cases of lymphosarcoma. Within a brief period these observations were confirmed and extended by investigators in Boston, Chicago, and Salt Lake City. Because of wartime secrecy these observations were not published immediately. They did not become generally known until 1946, when Gilman and Philips[2] presented their classic summary of the wartime work on the biological actions and therapeutic applications of the β-chloroethyl amines and sulfides. At the end of their presentation, these investigators made certain suggestions and comments, the substance of which is so pertinent to this conference that I should like to quote their concluding remarks in their entirety:

"It is possible that the potential value of the nitrogen mustards in the treatment of neoplastic diseases will be fully realized only when the opportunity to explore the relationship between chemical constitution and pharmaco-dynamic action has been exhausted. At present only two of the nitrogen mustards have been investigated clinically, namely, tris-(β-chloroethyl)-amine and methyl-bis-(β-chloroethyl)-amine. These have been the product of a screening program designed for the evaluation of toxic chemical warfare agents rather than of compounds of therapeutic interest. Literally hundreds of congeners remain to be synthesized and evaluated. Thus, a series of compounds which can reproduce in many ways the cellular effects of X-rays is available for chemical and biological investigation. It may be hoped that the previous successes which have characterized the evolution of chemotherapeutic agents by chemical alteration of a parent compound may be duplicated in the case of the β-chloroethyl amines. The result would be a compound having a sufficiently specific toxic action for certain types of proliferative cells to possess therapeutic value."

It has been more than ten years since this report directed the attention of the medical profession to the potentialities of nitrogen mustards and other alkylating agents in the treatment of neoplastic diseases. Numerous compounds have been synthesized and studied for basic biological activities. At least thirty, and probably more, agents have been brought to clinical trial. There are many who believe that it is time that some assessment be made of the accomplishments of these efforts. In what significant respects do the various alkylating agents differ from each other? What are their general and distinguishing chemical and biological features? What are their actual achievements and relative merits in the treatment of human neoplastic diseases? The answers to these questions are not clear at this time. The conference upon which this monograph is based was called for the purpose of refining such basic issues and, if possible, of indicating the desirability and direction of future work. If we address ourselves seriously to what has been accomplished, it is possible that we may clarify that which remains to be done.

References

1. Ehrlich, P. 1898. Über die Beziehungen von chemischer Constitution, Vertheilung und pharmakologischer Wirkung. Address before Verein für innere Medizin. *In* The Collected Papers of Paul Erhlich. **1**: 596–618. F. Himmelweit, Ed. 1956. Pergamon Press. London, England.
2. Gilman, A. & F. S. Philips. 1946. The biological actions and therapeutic applications of the β-chloroethyl amines and sulfides. Science. **103**: 409–415.

Part I. Chemistry of Alkylating Agents

FUNDAMENTAL MECHANISMS OF ALKYLATION

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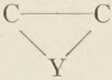
For the purposes of our discussion, alkylation may be defined as the conversion H—X → R—X. The source of the alkyl group, R, may be a simple alkylating agent, R—Y, from certain cyclic compounds, , or from an unsaturated compound, C=C. The alkylations of biological interest apparently all involve attachment of the alkyl group to X through an oxygen, nitrogen, or sulfur atom.

Starting with an alkylating agent, R—Y, where Y is a group stable as an anion ($Y^{\ominus}$), there are two generally recognized mechanisms for alkylation.[1] In one, so-called *first-order nucleophilic substitution*, S_N1, the reaction proceeds in two steps. The first step is a rate-controlling ionization of the R—Y bond, aided by a polar solvent that solvates and stabilizes the resulting ions. The second step is a rapid (and rather indiscriminate) reaction of the resulting alkyl carbonium ion (R^+) with a solvent molecule or some new anion (X^-):

$$\begin{array}{ll} R\text{—}Y \xrightarrow{\text{slow}} R^{\oplus}_{\text{solv.}} + Y^{\ominus}_{\text{solv.}} & \\ & (S_N1) \\ R^{\oplus} + X^{\ominus} \xrightarrow[\text{fast}]{\text{very}} RX & \\ (\text{or } R^{\oplus} + HX \rightarrow RX + H^{\oplus}) & \end{array}$$

The rate of this process is independent of the concentration of $X^{\ominus}$ (or HX) and depends on the concentration of the alkylating agent (and solvent):

$$\text{rate} = k_1[RY]$$

In the other mechanism, *second-order nucleophilic substitution*, S_N2, the bond-making and bond-breaking processes occur simultaneously:

$$X^{\ominus} + R\text{—}Y \xrightarrow{\text{slow}} X\text{—}R + Y^{\ominus} \quad (S_N2)$$

In this case the concentration and structure of $X^{\ominus}$ have a very important effect on the rate of the process:

$$\text{rate} = k_2[RY][X^{\ominus}]$$

In certain cases the reagent X can be a solvent molecule, such as water:

$$H_2O + R\text{—}Y \xrightarrow{k_w} HOR + Y^{\ominus} + H^{\oplus}$$

The relative effectiveness of various reagents $X^{\ominus}$ in displacing $Y^{\ominus}$ can most readily be compared experimentally by the ratio of their reactivity compared to that of water. Such a comparison of relative reactivities has been given by Swain[2] for the following reaction (see also TABLE 1).

TABLE 1

RELATIVE REACTIVITIES OF GROUPS X TOWARD METHYL BROMIDE

X	k/k_w	X	k/k_w	X	k/k_w
H_2O	1	$HPO_4^{\ominus}$	6,300	$SCN^{\ominus}$	6.3×10^4
$SO_4^{\ominus}$	300	$Br^{\ominus}$	8,000	$SH^{\ominus}$	1.3×10^5
$CH_3CO_2^{\ominus}$	500	$(NH_2)CS$	13,000	$S_2O_3^{\ominus}$	2.5×10^6
$Cl^{\ominus}$	1,000	$HO^{\ominus}$	16,000	$HPSO_3^{\ominus}$	4.0×10^6
C_5H_5N	4,000	$C_6H_5NH_2$	30,000		

Note the much greater value for $SH^{\ominus}$ than $OH^{\ominus}$, for thiosulfate than sulfate, and for thiophosphate than phosphate.

$$X^{\ominus} + CH_3Br \xrightarrow[25^\circ\ C.]{water} X\ CH_3 + Br^{\ominus}$$

The stability of $Y^{\ominus}$ as an anion has an important effect on the ease of the S_N2 process. The greater the stability of $Y^{\ominus}$, as predicted, for example, from the extent of dissociation of the acid, $H—Y \rightleftharpoons H^{\oplus} + Y^{\ominus}$, the greater the ease of its displacement.

TABLE 2

VARIATION OF REACTIVITY FOR THE REACTION $I^{\ominus} + RCl \rightarrow IR + Cl^{\ominus}$

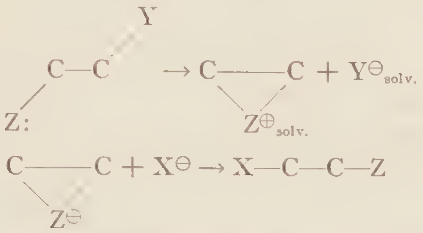
R	k_R/k_{n-Bu}	R	k_R/k_{n-Bu}
CH_3	120*		
C_2H_5	1.94		
$CH_3CH_2CH_2CH_2$	(1.00)		
$CH_3(CH_2)_{10}CH_2$	1.00		
$CH_3(CH_2)_{28}CH_2$	0.88		
$(CH_3)_2CH$	0.015	$CH_2{=}CH—CH_2$	80
$(CH_3)_2CHCH_2$	0.15*	$C_6H_5CH_2$	195
$(CH_3)_3C$	0.018	CH_3OCH_2	918
$(CH_3)_3CCH_2$	0.000026*	EtO_2CCH_2	2,800
		CH_3COCH_2	35,700
Cyclohexyl	$<.0001$	$C_6H_5COCH_2$	105,000

* For the reaction $Cl^{\ominus} + RBr \rightarrow RCl + Br^{\ominus}$ in acetone.[3]

The effect of R can be seen from a study of the replacement of one halogen by another (TABLE 2):[3, 4]

$$I^{\ominus} + R—Cl \xrightarrow[60^\circ\ C.]{acetone} I—R + Cl^{\ominus}$$

An important modification of the S_N2 reaction can occur when R contains a "neighboring group," Z, which can participate in the displacement process. This is most significant when Z is itself a nucleophilic group (such as RS— and R_2N—), with unshared electrons capable of forming a bond to carbon and when it is located so that the resulting cyclic intermediate has a strained and therefore reactive three-membered ring:

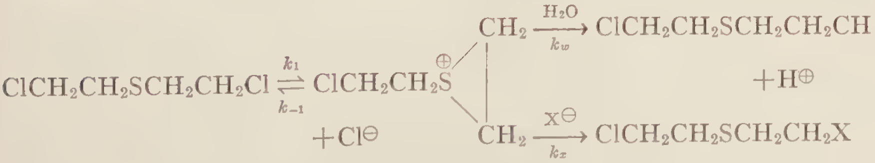


If the second step of this process is fast with respect to the first, the rate will be first order (like the S_N1 process), but the intermediate, instead of being a highly reactive and indiscriminate carbonium ion, will be a more stable ion capable of retaining a high degree of discrimination among reactants $X^{\ominus}$. For example, Ogston *et al.*[5] have measured the "competition factors" for a number of anions reacting with the cyclic intermediate from mustard gas.

TABLE 3
RELATIVE REACTIVITIES OF GROUPS X TOWARD MUSTARD GAS CYCLIC SULFONIUM ION

X	k_x/k_w*	pH
H_2O	1	—
$SO_4^{\ominus}$	250	—
$CH_3CO_2^{\ominus}$	500	7
Succinate	650	—
$Cl^{\ominus}$	1000	7
$(CH_2S^{\ominus})_2$	4.3×10^4	7
$SO_3^{\ominus}$	7.5×10^4	—
$S_2O_3^{\ominus}$	1.3×10^6	8
$[C_2H_5\overset{O}{\underset{S}{P}}S]^{\ominus}$	6.0×10^6	8

* The "competition factor" of Peters and Ogston, $k_x/k_w[H_2O]$, has been multiplied by the molar concentration of water (about 50).



The excellent agreement between relative reactivities of groups X for methyl bromide (TABLE 1) and for the cyclic sulfonium ion (TABLE 3) indicates that the product-controlling factor in the latter case must be very similar to that in the former. The greater reactivity of even un-ionized sulfur for the cyclic sulfonium salt is clearly indicated by the reaction of mustard gas with dilute aqueous solutions of thiodiglycol to react at the sulfur atom, not the oxygen:

TABLE 4

COMPARISON OF RATE CONSTANTS FOR SOME β-CHLOROETHYLAMINES ($ClCH_2CH_2NRR'$) IN 2:1 ACETONE-WATER SOLUTION

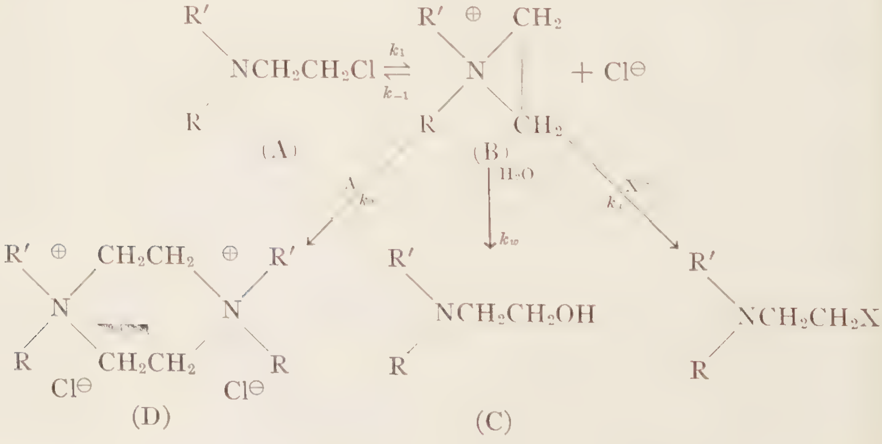
R	R′	ΔpK_a*	Main reaction†	Rate constants (min.$^{-1}$)			
				k_1	k_{-1}	k_2	k_w
C_2H_5	C_2H_5	−2.66	I	.202	<.02	<1.0	<.05
ClC_2H_4	CH_3 (HN-2)	−4.76	D	.02	1.35	.4	.0012
ClC_2H_4	C_2H_5 (HN-1)	−4.87	H	.085	1.5	.08	.0057
ClC_2H_4	ClC_2H_4 (HN-3)	−7.18	H	.0055	∼200	≤20	∼1

* Decrease in base strength of the amine.

† I = internal cyclization to imonium ion (B); D = dimerization to (D); H = hydrolysis to (C).

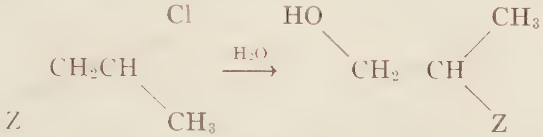
$$S(CH_2CH_2Cl)_2 + 2S(CH_2CH_2OH)_2 \xrightarrow[\text{soln.}]{\text{water}} [(HOCH_2CH_2)_2\overset{\oplus}{S}CH_2CH_2]_2S$$

In the case of nitrogen mustard gases, the cyclic intermediate is less reactive, and has even been isolated as the picrylsulfonate salt.[6] Bartlett *et al.*[7] have made a detailed study of the kinetics for several nitrogen mustards, as summarized in TABLE 4:



The selectivity of the cyclic imonium ion (B) for reactions in aqueous media is indicated by the accuracy of analysis for (B) by reaction with thiosulfate[6] or thiophosphate[7] ions (X). The much greater selectivity of the intermediate (B) for un-ionized nitrogen rather than un-ionized oxygen is indicated by the high yield of dimer (D) from HN-1 in ethanol. No ethoxyl compound was isolated.

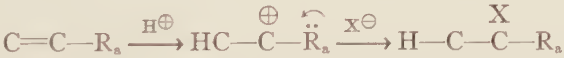
One consequence of "neighboring group" participation is the possibility for rearrangements during replacement:



This type of rearrangement, predictable on the basis of a cyclic intermediate ion, has been shown for the case of Z = —SR[8] and Z = —NR$_2$.[9]

It seems reasonable to believe that ethylenimine and ethylene oxide derivatives behave in a manner similar to the imonium and sulfonium ions, but quantitatively less rapidly. The same order of reactivity seems to prevail, since reaction at sulfur or nitrogen predominates over reaction at oxygen, even in dilute aqueous solution.

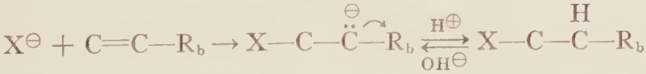
Alkylation using unsaturated compounds containing a carbon-carbon double bond as the alkylating agents also involves a duality of mechanism, in this case depending largely on the nature of the group or groups attached to the double bond. If the group (R$_a$) is an electron-donating group, such as alkyl, aryl, or alkoxyl, the addition is acid-catalyzed, is initiated by electrophilic attack by a proton, and proceeds through a carbonium ion intermediate:



$$C{=}C{-}R_a \xrightarrow{H^{\oplus}} HC{-}\overset{\oplus}{C}{-}\ddot{R}_a \xrightarrow{X^{\ominus}} H{-}C{-}\overset{X}{C}{-}R_a$$

This type of addition involves an intermediate similar to that of the S$_N$1 reaction. It would appear to be less discriminating in its reactions and of less physiological interest.

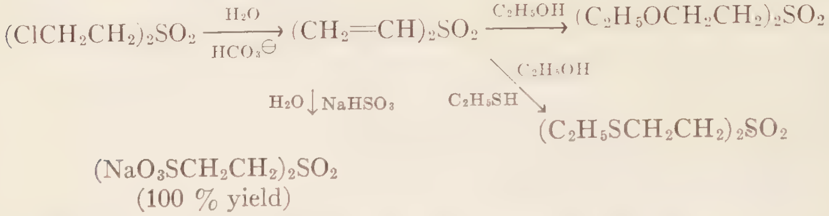
If the group attached to the double bond (R$_b$) is electron-attracting (—NO$_2$, —SO$_2$R, —COR, —CO$_2$R), the addition proceeds under basic (or even neutral) conditions, is initiated by attack of a nucleophilic reagent X$^{\ominus}$, and proceeds through a carbanion intermediate:



$$X^{\ominus} + C{=}C{-}R_b \rightarrow X{-}C{-}\overset{\ominus}{\ddot{C}}{-}R_b \underset{OH^{\ominus}}{\overset{H^{\oplus}}{\rightleftharpoons}} X{-}C{-}\overset{H}{C}{-}R_b$$

An interesting feature of these reactions is that they are reversible, particularly under basic conditions.

An interesting example of this class of compounds is mustard sulfone, (ClCH$_2$CH$_2$)$_2$SO$_2$. This material is completely stable to hydrolysis in distilled water for weeks, but liberates chloride ion copiously in five minutes in bicarbonate buffered aqueous solution.[10] Products of alkylation by mustard sulfone are formed in basic solution and evidently all proceed through primary dehydrochlorination to vinyl sulfone.[11]



$$(ClCH_2CH_2)_2SO_2 \xrightarrow[HCO_3^{\ominus}]{H_2O} (CH_2{=}CH)_2SO_2 \xrightarrow{C_2H_5OH} (C_2H_5OCH_2CH_2)_2SO_2$$

$$(CH_2{=}CH)_2SO_2 \xrightarrow[C_2H_5OH]{C_2H_5SH} (C_2H_5SCH_2CH_2)_2SO_2$$

$$(CH_2{=}CH)_2SO_2 \xrightarrow{H_2O,\ NaHSO_3} (NaO_3SCH_2CH_2)_2SO_2$$

(100 % yield)

The ease of addition of compounds capable of forming the sulfur-carbon bond, in preference to the carbon-oxygen bond, is again a feature of the chemical characteristics of this reaction. Similarly, for additions to vinyl ketones and α,β-unsaturated esters (R_b = –COR or –CO_2R), addition of amine occurs preferentially even in alcoholic or aqueous solutions:

$$R_2NH + CH_2{=}CHCOR' \xrightarrow[R''OH]{} R_2NCH_2CH_2COR'$$

In summary, it appears that for the alkylation of many H–X compounds to give R—X, with a wide variety of alkylating agents, the reactions proceed preferentially for alkylation on sulfur, then nitrogen, and least readily on oxygen. In every case, the ease of alkylation may be materially enhanced by prior ionization to $X^{\ominus}$.

References

1. INGOLD, C. K. 1953. Structure and Mechanism in Organic Chemistry. Chap. VII. Cornell Univ. Press, Ithaca, N. Y.
2. SWAIN, C. G. 1953. Abstr. 13th Natl. Org. Chem. Symposium, Am. Chem. Soc. : 65.
3. CONANT, J. B., W. R. KIRNER & R. E. HUSSEY. 1925. J. Am. Chem. Soc. **47**: 488.
4. HUGHES, E. D., C. K. INGOLD & J. D. H. MACKIE. 1955. J. Chem. Soc. : 3173.
5. OGSTON, A. G., E. R. HOLIDAY, J. ST. L. PHILPOT & L. A. STOCKEN. 1948. Trans. Faraday Soc. **44**: 45.
6. GOLUMBIC, C., J. S. FRUTON & M. BERGMANN. 1946. J. Org. Chem. **11**: 518.
7. BARTLETT, P. D., S. D. ROSS & C. G. SWAIN. 1949. J. Am. Chem. Soc. **71**: 1415.
8. FUSON, R. C., C. C. PRICE & D. M. BURNESS. 1946. J. Org. Chem. **11**: 477.
9. ROSS, S. D. 1947. J. Am. Chem. Soc. **69**: 2982.
10. PRICE, C. C. & O. H. BULLITT, JR. 1947. J. Org. Chem. **12**: 238.
11. KRETOV, A. E. 1930. J. Russ. Phys. Chem. Soc. **62**: 1.

IN VITRO REACTIONS OF BIOLOGICAL ALKYLATING AGENTS

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Examples of most of the types of compound to be dealt with in this monograph are shown in FIGURE 1. A wide variety of structures is involved; the only common feature is an ability to function as an alkylating agent under mild conditions in aqueous solution, that is, under physiological conditions. In view of this variety of structures it seems extremely unlikely that these compounds are producing their characteristic effects by any process of physical adsorption or competitive inhibition. This point is emphasized by the correlation between chemical reactivity and biological effectiveness. In this paper I shall regard a compound as being biologically active if it has been shown to inhibit the growth of the transplanted Walker rat carcinoma. FIGURES 2 and 3 show examples of closely related compounds the activity of which is lost if chemical reactivity is reduced. The reactivity of the epoxides shown in FIGURE 2 is assessed by the rate at which they react with the thiosulphate ion (Ross, 1950); the reactivity of the aromatic nitrogen mustards shown in FIGURE 3 is assessed by the rate of hydrolysis under standard conditions (Ross, 1953).

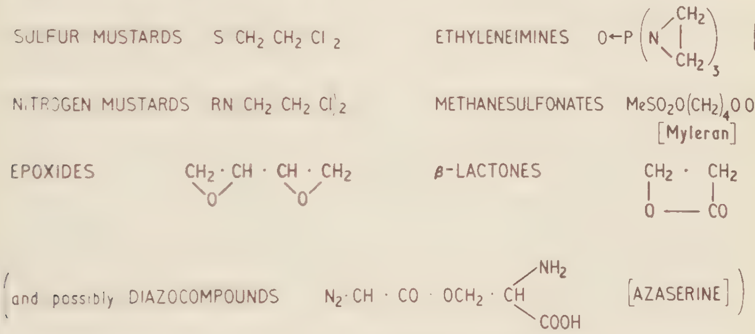


FIGURE 1. Biological alkylating agents.

We can justifiably assume that the compounds are biological alkylating agents; now we must consider which chemical groupings can react *in vivo.* The two mechanisms of alkylation that we need to examine are shown in FIGURE 4: the unimolecular mechanism, in which the rate-determining step is the preliminary fission of a covalent bond; and the bimolecular mechanism, which involves the attack of the agent by a nucleophilic, that is, electron-rich center. These two mechanisms are illustrated by reference to aromatic nitrogen mustards and sulfonic esters. The other alkylating agents generally react by a combination of these mechanisms, depending on conditions of *p*H and reactant concentration. A feature of the unimolecular (or S_N1) type of reaction is the fact that the rate at which the reagent reacts is dependent on the rate of fission of the covalent linkage (that is, in the case of a mustard gas derivative, the rate of ionization), and this is determined by the nature of the medium. It is largely independent of the concentration of reacting centers. On the other hand, the rate of combination of an agent that reacts by a bimolecular (or S_N2) mechanism is entirely dependent on the concentration of reacting centers. This distinction is likely to be of importance in biological systems where one has a limited amount of agent diffusing through regions in which the concentration of reacting groups is not constant.

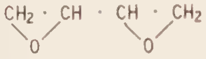
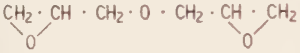
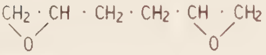
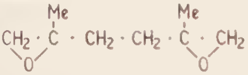
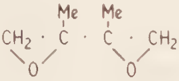
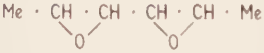
COMPOUND	RELATIVE REACTIVITY (K_{Thio})	BIOLOGICAL ACTIVITY
 $CH_2\cdot CH\cdot CH\cdot CH_2$	85	+
 $CH_2\cdot CH\cdot CH_2\cdot O\cdot CH_2\cdot CH\cdot CH_2$	69	+
 $CH_2\cdot CH\cdot CH_2\cdot CH_2\cdot CH\cdot CH_2$	40	+
 $CH_2\cdot C(Me)\cdot CH_2\cdot CH_2\cdot C(Me)\cdot CH_2$	23	—
 $CH_2\cdot C(Me)\cdot C(Me)\cdot CH_2$	21	—
 $Me\cdot CH\cdot CH\cdot CH\cdot CH\cdot Me$	2·3	—

FIGURE 2

By either mechanism the effective reaction is the displacement of one group attached to a carbon atom by another, and it is convenient, if not strictly accurate in a kinetic sense, to picture the reaction as involving a carbonium ion in all cases, as in the examples shown in FIGURE 5 (compare Hammett, 1940). This positively charged center will react with any electron-rich centers in biological systems.

Next we must consider the types of chemical groups that can react with these positively charged carbonium ions or, in other words, the type of group that is nucleophilic or electron-rich. The main types of nucleophilic centers encountered in biological material are organic and inorganic anions, amino groups, and sulfide groups. FIGURE 6 shows the products formed when such groups react. In this way, esters, alkylated amines, ethers, thio ethers, and ammonium and sulfonium compounds are formed. Under physiological

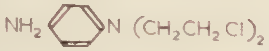
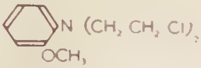
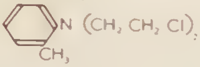
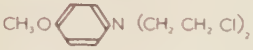
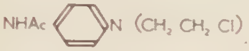
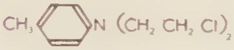
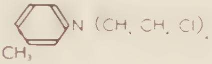
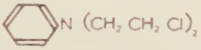
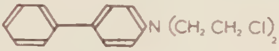
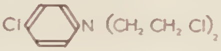
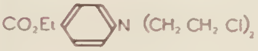
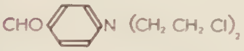
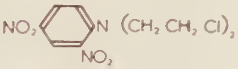
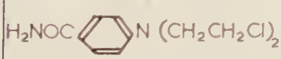
COMPOUND	% HYDROLYSIS IN $\frac{1}{2}$ HR IN 50% ACETONE AT 66°	BIOLOGICAL ACTIVITY
	100	+
	89	+
	83	+
	58	+
	41	+
	38	+
	21	+
	20	+
	12	−
	9	−
	< 1	−
	< 1	−
	< 1	−
	< 1	−

FIGURE 3

MUSTARD S_N1

$$R_2\cdot N\cdot CH_2\cdot CH_2\cdot Cl \rightleftharpoons R_2\cdot N\cdot CH_2\cdot CH_2^+ + Cl^-$$

$$R_2\cdot N\cdot CH_2\cdot CH_2^+ + A^- \rightarrow R_2\cdot N\cdot CH_2\cdot CH_2\ A$$

SULFONIC ESTER S_N2

$$R\cdot OSO_2\cdot R' + A^- \rightarrow \left[\overset{\delta-}{A}\cdots R\cdots \overset{\delta-}{OSO_2}\cdot R'\right]$$

$$\rightarrow R\cdot A + \bar{O}SO_2\cdot R$$

FIGURE 4. Unimolecular and bimolecular displacements.

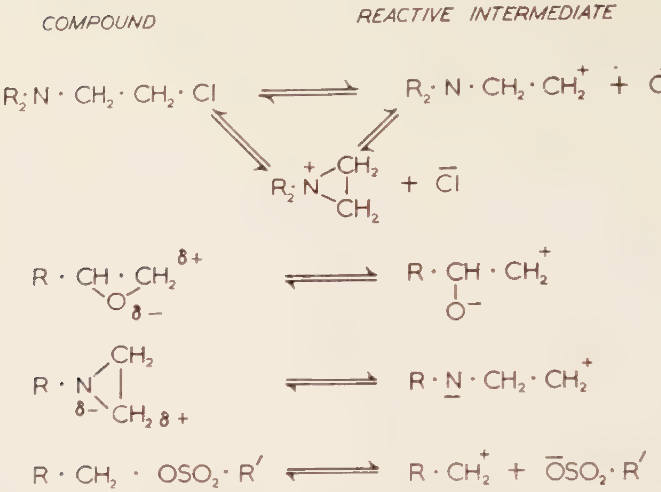
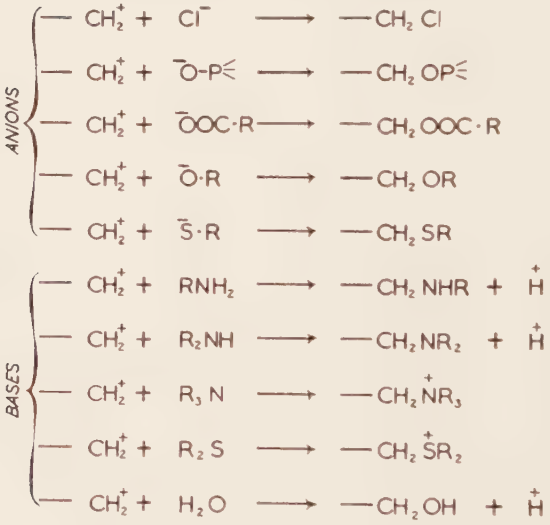


FIGURE 5. Reaction mechanisms.



THERE WILL BE NO REACTION WITH UNDISSOCIATED ACID GROUPS (RCO_2H) OR AMMONIUM CATIONS. (RNH_3^+)

FIGURE 6. Reaction of carbonium ions.

conditions there will be little or no reaction with undissociated acid groups or with ammonium cations, since these are not nucleophilic.

It is obvious that whether a particular group is in a form capable of reacting at physiological *p*H will depend on the dissociation constant of that group. By way of illustration, let us consider the functional groups in two important biological materials, the proteins and the nucleic acids. In doing this we do not imply at this stage that reaction with these materials is responsible for the biological effects. TABLE 1 shows the fraction (f) of the various centers that will be in the reactive form at pH 7.5. It will be seen that the reactive groups in proteins will be the carboxyl groups, the sulfhydryl groups in terminal cysteinyl units, as well as the histidine and terminal α-amino groups. In nucleic acids, reaction could occur with the primary and secondary phosphoryl groups and with the so-called aromatic-type amino groups. FIGURES 7 and 8 are diagrammatic representations of segments of proteins and nucleic acids to show the location of the reactive groups. I should call attention to the difference in reactivity to be expected between terminal and nonterminal cysteine units (compare Benesch and Benesch, 1955).

TABLE 1

pK$_a$ VALUES OF ACIDIC AND BASIC GROUPS IN PROTEINS AND IN NUCLEIC ACIDS

Group	pK$_a$	Fraction (f) of groups in reactive form at pH 7.5
Proteins		
α-Carboxyl	3.0–3.2	0.9999
Carboxyl (aspartyl)	3.0–4.7	0.9999–0.999
Carboxyl (glutamyl)	4.4	0.999
Phenolic hydroxyl (tyrosine)	10.4	0.001
Sulfhydryl (terminal cysteinyl)	7.9–8.5	0.1–0.06
Sulfhydryl (nonterminal cysteinyl)	10.8	5×10^{-4}
Imidazolium (histidine)	5.6–7.0	0.99–0.76
α-Ammonium	7.6–8.4	0.44–0.11
ϵ-Ammonium (lysine)	9.4–10.6	10^{-2}–10^{-3}
Guanidinium (arginine)	11.6–12.6	10^{-4}–10^{-5}
Nucleic acids		
Primary phosphoryl	2.0	0.9999
Secondary phosphoryl	6.0	0.96
Aromatic hydroxyl (uracil, thymine)	10.2	0.002
Aromatic hydroxyl (guanine)	10.1	0.0025
Sugar hydroxyl	13	10^{-5}
Aromatic amino (guanine)	2.3	0.9999
Aromatic amino (adenine, cytosine)	3.7–4.2	0.999

There is a wide variation in the rate at which even the reactive forms of the various groups combine with alkylating agents. This affinity or nucleophilic capacity of a center is conveniently assessed by what Ogston (1948) has called the "competition factor" of the group. The value of the competition factor (F) shown in TABLE 2 relates to the rate at which the center is alkylated under standard conditions. High values for F indicate a great affinity between the group and an alkylating agent. It will be seen that sulfur-containing anions have by far the greatest affinity. The values for organic anions fall in the range 1 to about 50. The zero value for glucose shows, as would be expected, that there is no appreciable reaction with

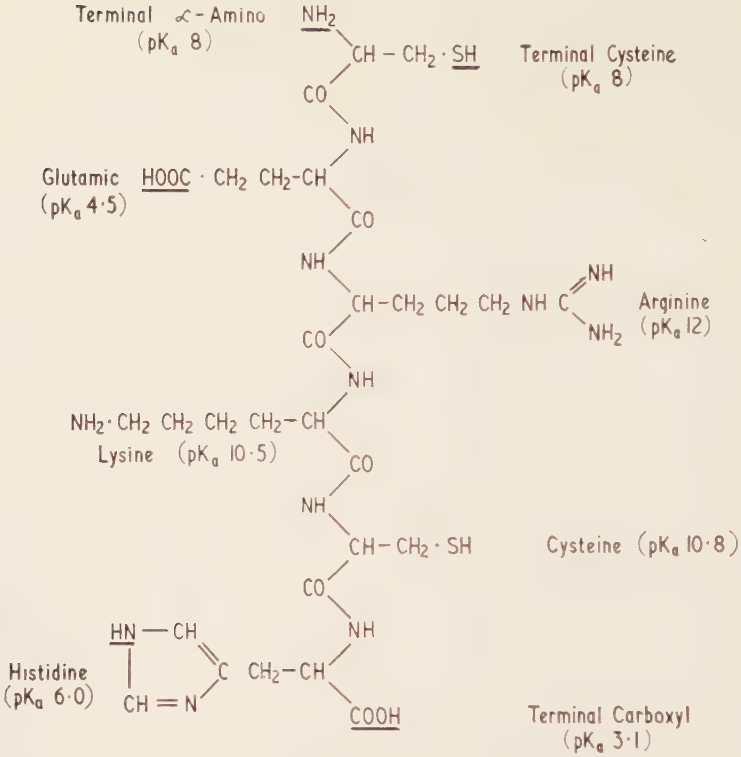


FIGURE 7. Reactive groups in proteins.

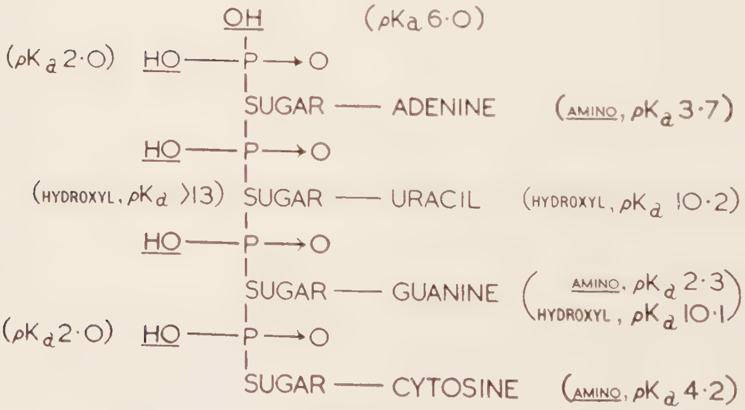


FIGURE 8. Reactive groups in nucleic acids.

aliphatic hydroxyl groups at *p*H 7. Bases have relatively high competition factors at a *p*H where they are present mainly in the nonionic form (compare the values for methylamine and dimethylamine at *p*H 13), but the value is much lower at a *p*H where the cationic form predominates (see the value for trimethylamine at *p*H 7). The effective competition factor of a center at a specified *p*H is given by the product of the competition factor of the reactive form (*F*) and the proportion (*f*) of the total groups in that form. Some values of effective competition factors at *p*H 7.5 are given in TABLE 3.

TABLE 2
COMPETITION FACTORS*

Substance	*F*	*p*H
Dithiophosphate $(PS_2O_2)'''$	1.3×10^5	7
Thiophosphate $(PSO_3)'''$	3.8×10^4	8
Thiosulphate	2.7×10^4	8
Cysteine ethyl ester	1.7×10^3	7
Methylamine	3.9×10^2	13
Dimethylamine	2.55×10^2	13
Phosphate	7.5×10^1	8
α-Glycerol phosphate	5.4×10^1	8
Oxalate	4.1×10^1	7
Malonate	3.2×10^1	7
Chloride	2.1×10^1	7
Acetate	1.0×10^1	7
Trimethylamine	6.7	7
Sulfate	4.9	7
Formate	3.0	7
Lactate	1.1	7
Nitrate	0.2	7
Glucose	0	7

* From Ogston (1948).

TABLE 3
EFFECTIVE COMPETITION FACTORS (*Ff*) OF REACTIVE GROUPS

Group	*F*	*f*	*Ff*
Terminal cysteinyl	10^5	10^{-1}	10^4
Aromatic amino	10^2	1	10^2
Phosphoryl	50–100	1	50–100
Carboxyl	10–80	1	10–80
Imidazole	70	1–0.8	70–56
Nonterminal cysteinyl	10^5	10^{-4}	10
Aliphatic amino	4×10^2	10^{-2}–10^{-3}	4–0.4

The relative amount of any group reacting is proportional $F \times f \times c$; where c is the concentration of that group in the system.

Another factor that determines the extent to which a particular group will react is the concentration *(c)* of that group in the system. This is of importance in tumor inhibition studies, where a limited amount of agent is available for combination with a large excess of reacting groups. The expression $F \times f \times c$ at the bottom of the table refers to homogeneous solutions containing relatively simple molecules. We must now consider some factors that could modify the conclusions drawn. Alexander (1954) reported that the apparent competition factor of carboxyl groups in polymethacrylic acid is considerably greater than that for the carboxylate ions from acetic acid. The explanation is almost certainly that adsorption of the agent onto the polymer increases the concentration factor (*c*). This will clearly be an important effect in biological systems, and there is ample evidence for it. For example, it has been found that the competition factors for serum albumin and nucleic acids are considerably higher toward mustard gas than could be accounted for by the values for the constituent groups in homogeneous solutions. Again, Barron *et al.* (1948) showed that high concentrations of thiosulfate (a very reactive anion) were required to protect choline oxidase against inactivation by HN2; this also indicates high affinity between the enzyme and the mustard. Friedenwald (1951) observed the opposite effect, in that glutathione is much less reactive toward mustard gas in intact corneal epithelium than it is in the homogenized tissue or in aqueous solution. Elsewhere in this monograph (p. 682) K. A. Stacey refers to an increase in the reactivity of SH groups when a protein is denatured. This may be due to a steric factor, for sulfhydryl groups are often present on short cysteinyl chains in peptides and proteins and, in folded structures, they may not be as accessible for reaction as other groups.

Another complication is the fact that groups in association may have altered reactivity. Alexander (1954) and Davis and Ross (1952) have suggested that the unexpectedly high reactivity of amino groups in some proteins is due to the salt linkage with a carboxyl group having the structure —NH_2 . . . HOOC— rather than $—\overset{+}{N}H_3 \ldots \overset{-}{O}OC—$. In the first form the amino group is uncharged and therefore reactive.

From our considerations, my associates and I have reached the conclusion that ionized acid groups and sulfhydryl groups, as well as free amino groups, are likely centers for reaction with biological alkylating agents. I should now like to present some speculations that in my opinion tend to limit the site of reaction that may be responsible for the tumor-growth inhibitory effect. A large number of compounds with higher reactivity toward amino groups than to acidic groups have been tested by Hendry *et al.* (1951) and in our laboratories. Some of these compounds are shown in FIGURE 9. Both teams were unable to demonstrate any appreciable antitumor effects with these compounds. Furthermore, a wide range of agents more reactive toward —SH groups has been examined over the years at the Chester Beatty Research Institute (some of the types are shown in FIGURE 9), and none of these compounds has proved to be an effective inhibitor of the Walker rat carcinoma. By a process of elimination we are left with the ionized acid groups and, while we should not overemphasize evidence based on elimination reasoning, I suggest that we are justified in forming the working hypothesis that biological alkylating agents produce their antitumor effects by esterifying ionized acid groups. On the basis of this hypothesis we can proceed to a consideration of how such a reaction could be expected to interfere with processes of cell division for we must not forget that the dividing cell is more susceptible to the action of these agents than a resting cell.

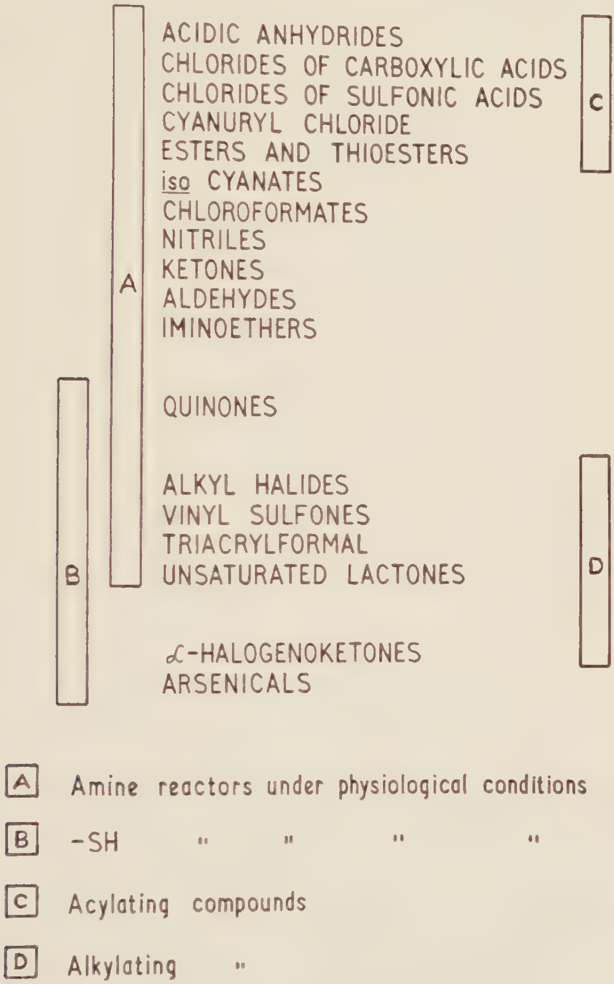
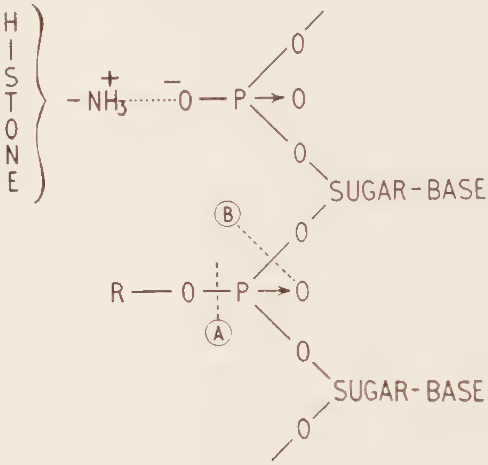


FIGURE 9. Amine and sulfhydryl group reactors.

There are reasons for believing that reaction with nucleic acids may be responsible for the biological effects. For instance, Herriott (1948) has shown that the *Pneumococcus* transforming principle, composed mainly of deoxyribonucleic acid, exhibits the greatest sensitivity toward mustard gas of all the materials he examined, being inactivated by a 2-hr. exposure to 6×10^{-5} molar mustard gas. Zamenhof *et al.* (1956) found that the *Hemophilus influenzae* transforming principle was very sensitive to the action of alkylating agents—exposure for 6 hr. to 2×10^{-5} molar HN2 (nitrogen mustard gas) causing 90 per cent inactivation. On the basis of finding a correlation within a limited series of nitrogen mustards between the activity on the transforming principle and on the Walker tumor, these investigators have provisionally suggested that the carcinostatic action may be due to inactivation of cellular deoxyribonucleic acid. Another indication comes from the finding (Loveless and Revell, 1949; Revell, 1953) that, when chromosome aberrations are produced in dividing cells of *Vicia* root tips by the action of alkylating agents, the position of "breaks" is not as random as with radiation, but is more localized, often in the heterochromatic regions, that is, in regions of high nucleic acid content. Thus there is evidence that biologically active nucleic acids are very sensitive to the action of alkylating agents.

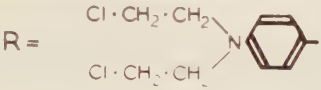


R is a radical from an alkylating agent.

FIGURE 10

In vitro experiments have shown that alkylating agents will react with nucleic acids and cause considerable changes in their physical properties, for example, in the shape of the macromolecule and in its ability to combine with basic material. Esterification of the phosphate groups would be expected to reduce the ability of a nucleic acid to associate with basic protein (FIGURE 10); Alexander (1951) has found that the biologically more active polyfunctional compounds are more effective in this respect than monoalkylating agents. Reaction of the primary phosphate groups of nucleic acids with alkylating agents would produce less stable triesters that on hydrolysis at the sugar-phosphate linkages, would result in the breakdown of the nucleic acid structure. This reaction and its consequences are discussed by Stacey elsewhere in this monograph. Either of these actions of the alkylating agents would be expected to have a profound effect on the process of cell division, especially if chromosomal nucleic acid were involved.

There is also the possibility that these agents produce their characteristic effects by enzyme inhibition; in this connection, phosphokinases, certain peptidases, choline oxidase, and acetylcholine esterase have proved to be among the most sensitive enzymes so far examined (Needham, 1948).



Compound	% Hydrolysis in $\frac{1}{2}$ hr. in 1:1 acetone-water at 66°	Biological activity
$R\cdot COOH$	15	±
$R\cdot CH_2\cdot COOH$	39	++
$R\cdot CH_2\cdot CH_2\cdot COOH$	41	+
$R\cdot O\cdot CH_2\cdot COOH$	48	+
$R\cdot CH_2\cdot CH_2\cdot CH_2\cdot COOH$	42	+++
$R\cdot O\cdot CH_2\cdot CH_2\cdot COOH$	52	+++
$R\cdot CH_2\cdot CH_2\cdot CH_2\ CH_2\cdot COOH$	39	±
$R\cdot O\cdot CH_2\cdot CH_2\cdot CH_2\cdot COOH$	50	+
$R\cdot O\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH_2\cdot COOH$	44	++

FIGURE 11. Variation in biological activity in a series of acidic derivatives of comparable reactivity.

However, the objection to the enzyme inhibition theory has been that the concentrations of agent required for inactivation do not appear to be achieved *in vivo* when cytotoxic effects can be demonstrated (Philips, 1950).

We have seen that the group of alkylating agents presently under discussion has many features in common, and it might be wondered whether there is any prospect of obtaining selectivity of action in a particular type of tissue. It is our belief that this can be achieved. F. Bergel deals with this aspect elsewhere in these pages (p. 1238), but I shall indicate very briefly, by reference to some of our work on the aromatic nitrogen mustards, how we are tackling the problem. We have modified the physical properties and transport characteristics of these compounds by incorporating water or fat solubilizing groups, by rendering the molecule anionic or cationic, and by attaching amino acid side chains. The importance of the carrying structure is illustrated in FIGURE 11, where a variation in biological activity is shown in a series of acidic derivatives of comparable reactivity. Maximum activity is exhibited by the phenylbutyric acid derivative (CB 1348 or chlorambucil) and by the isosteric phenoxypropionic derivative. Another line of approach consists of the preparation of compounds that would be expected to be inactive because of low chemical reactivity, but that could be converted into a reactive compound by a process known to occur *in vivo*. One example of this type is the azo compound shown in FIGURE 12, which we are convinced becomes activated by *in vivo* reduction of the azo linkage, since it was shown (Ross and Warwick, 1955; 1956) that substituents that facilitate reduction enhance biological activity. One would hope that this reductive activation would not take place equally well at all sites and that some specificity of action would result.

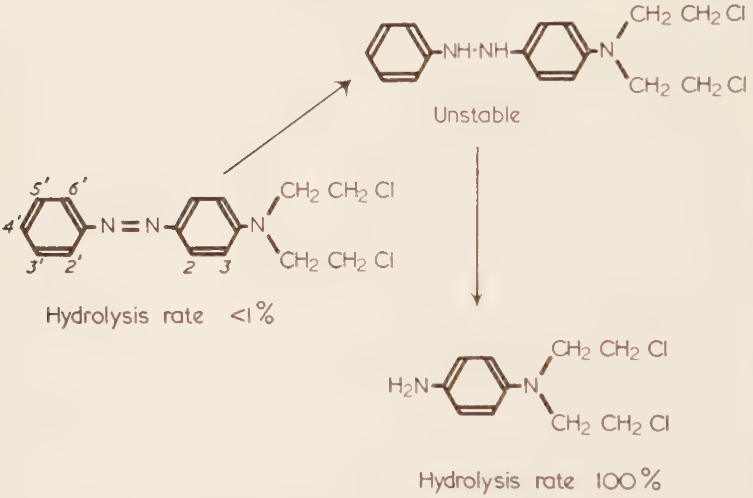


FIGURE 12. Reductive activation of chloroethylamino-azobenzenes.

In conclusion, it can be said that we have achieved some specificity of action with our transport variants, particularly with respect to various blood elements, as covered by L. A. Elson elsewhere in this monograph (p. 826). Some of the azo compounds are powerful inhibitors of the Walker rat carcinoma at doses that do not cause the serious bone marrow damage normally associated with the administration of nitrogen mustards.

References

ALEXANDER, P. 1952. Nature. **169**: 226.
ALEXANDER, P. 1954. Advances in Cancer Research. **2**: 1.
BARRON, E. S. G., G. R. BARTLETT & Z. B. MILLER. 1948. J. Exptl. Med. **87**: 489.
BENESCH, R. E. & R. BENESCH. 1955. J. Am. Chem. Soc. **77**: 5877.
DAVIS, W. & W. C. J. ROSS. 1952. J. Chem. Soc. : 4296.
FRIEDENWALD, J. S. 1951. Ann. N. Y. Acad. Sci. **51**(8): 1432.

HAMMETT, L. P. 1940. Physical Organic Chemistry : 291. McGraw-Hill. New York, N. Y.

HENDRY, J. A., F. L. ROSE & A. L. WALPOLE. 1951. Brit. J. Pharmacol. **6**: 201.

HERRIOTT, R. M. 1948. J. Gen. Physiol. **32**: 221.

LOVELESS, A. & S. REVELL. 1949. Nature. **164**: 938.

NEEDHAM, D. M. 1948. Biochem. Soc. Symposia Cambridge, Engl. **2**: 16.

OGSTON, A. G. 1948. Trans. Faraday Soc. **44**: 45.

PHILIPS, F. S. 1950. J. Pharmacol. Exptl. Therap. **99**: 281.

REVELL, S. 1953. Heredity. **6** (Suppl.) : 107.

ROSS, W. C. J. 1950. J. Chem. Soc. : 2257.

ROSS, W. C. J. 1953. Advances in Cancer Research. **1**: 397.

ROSS, W. C. J. & G. P. WARWICK. 1955. Nature. **176**: 298.

ROSS, W. C. J. & G. P. WARWICK. 1956. J. Chem. Soc. : 1364, 1719, 1724.

ZAMENHOF, S., G. LEIDY, E. HAHN & H. E. ALEXANDER. 1956. J. Bacteriol. **72**: 1.

THE REACTIONS OF THE "RADIOMIMETIC" ALKYLATING AGENTS WITH MACROMOLECULES *IN VITRO*

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Introduction

There can be no doubt that the biological activity of the "radiomimetic" alkylating agents is manifested in the cell by the one property they have in common--reaction with nucleophilic groups. Since there are so many possible sites for reaction, much if not most of the reagent is wasted in the cell in reactions that are biologically harmless. The determination of which cell constituents have reacted with any one agent is not in itself sufficient to establish which of these reactions is responsible for the biochemical lesion that ultimately leads to the biological effect. It seems likely, in view of the small amount of material required to produce a marked biological effect, that alkylation of the macromolecules in the cell is involved. *In vitro* studies can establish the most likely site of reaction and some of the possible consequences of these reactions. The results to be reported in this paper have been obtained from experiments designed to answer the following questions:

(1) What are the relative extents of reaction of the various nucleophilic groups in these macromolecules, particularly the proteins and nucleic acids?

(2) What changes in the macromolecules result from alkylation?

(3) In what respects do the bifunctional reagents differ from the monofunctional analogues whose biological activity is so much less?

Alexander[1] has summarized a number of papers published on the reactions of sulfur mustard [$S\cdot(CH_2\cdot CH_2\cdot Cl)_2$] with nucleic acids and proteins, but much of this work was done in the presence of an enormous excess of reagent, so that it is impossible to assess the relative reactivities of the different groups when all the accessible sites have been alkylated. Much less data are available concerning the reactions of the other classes of alkylating agents, nitrogen mustards, epoxides, ethylenimines, and mesyloxy compounds, all of which have radiomimetic properties.[2] In this paper we are concerned with a representative range of these compounds and their reactions with proteins, nucleic acids, and nucleoproteins when the concentration of alkylating agent is considerably below that required to react with all the available nucleophilic centers. Attention is directed, therefore, to the predominant reactions when there is competition for a limited quantity of alkylating agent, since these are the conditions under which these drugs react in the cell.

Sites of Reaction in Proteins

Carboxyl and amino groups. Bovine serum albumin* was reacted under physiological conditions with the alkylating agents shown in TABLE 1. The

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* Armour & Co., Chicago, Ill.

TABLE 1
CHANGES PRODUCED IN SERUM ALBUMIN BY RADIOMIMETIC AGENTS*

Treatment	Per cent reduction			
	Carboxyl groups	Amino groups	Imidazole groups	Tyrosine
$O-[CH_2\cdot \underset{O}{CH-CH_2}]_2$	27	46	77	No reaction
$CH_3\cdot \underset{O}{CH-CH_2}$	22	60	70	No reaction
Myleran	31	0	42	No reaction
Dimethyl Myleran	16	5	12	No reaction
Chlorambucil	25	7	5	No reaction

* Three per cent protein solution +0.12 moles of reagent.

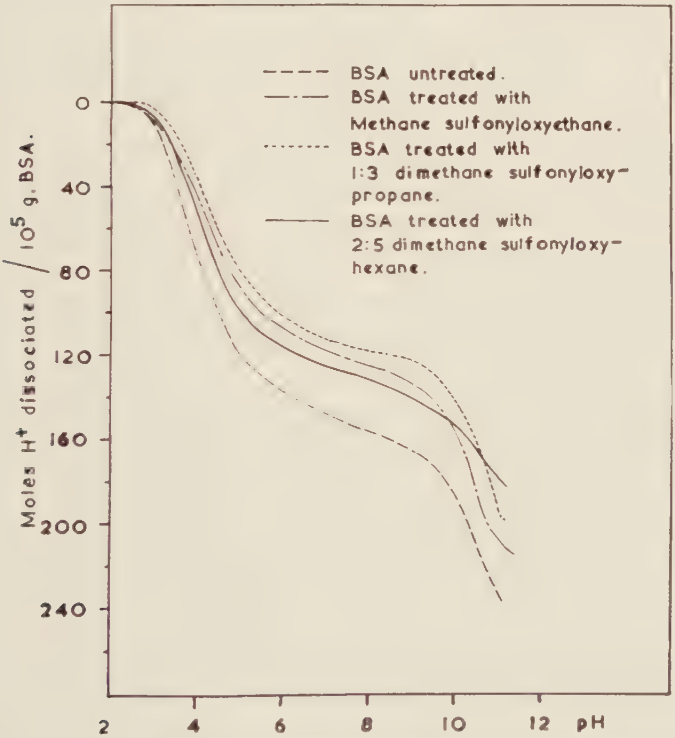
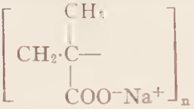
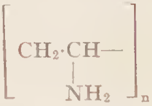


FIGURE 1. Change in the titration curve of bovine serum albumin after treatment with some radiomimetic alkylating agents. In every case the number of groups titrating between *p*H 2 and *p*H 5.5 is reduced by treatment; this change provides a direct measure of the number of carboxl groups that have been esterified.

extent of reaction with the carboxyl groups was obtained from the change in the number of groups that titrate between pH 2 to 5.5 (FIGURE 1), with the exception of the nitrogen mustards, for which a more indirect method of estimation was used, since they introduce groups that titrate in this range. The numbers of primary amino groups were measured by the standard Van Slyke method, and the imidazole groups (the side chains of histidine) were estimated colorimetrically. The marked change in the ultraviolet absorption spectrum of tyrosine that occurs upon ionization, when the pH is changed from 7 to 12, is prevented by alkylation, so that a comparison of the spectra under these two conditions can be used to measure reaction with tyrosine.

TABLE 2
INTERNAL CROSSLINKING OF POLYBASE AND POLYACID*

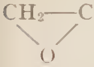
Alkylating agent	 $\left[CH_2\cdot\overset{CH_3}{\underset{COO^-Na^+}{C}}— \right]_n$		 $\left[CH_2\cdot\underset{NH_2}{CH}— \right]_n$	
	Per cent esterification	Per cent change in viscosity	Per cent reaction	Per cent change in viscosity
$X\cdot(CH_2)_4\cdot X$	7	−45	0	0
$X\cdot CH_2\cdot C{=}C\cdot CH_2\cdot X$	26	−80	16	−20
HN2	50	−50	19	−25
Bisepoxide	24	0	28	−37
Trisacryl formal	0	0	50	−38

* Polymer concentration, 0.2 N (about 1.5 per cent); reagent, 0.05 M (that is, maximum esterification possible, 50 per cent).

TABLE 1 shows that, while all the different types of alkylating agents esterify carboxyl groups, only the epoxides react extensively with the primary amino and imidazole groups provided by the side chains of lysine and histidine, respectively. Experiments with two model substances that have only one kind of reactive group, the polyacid, polymethacrylic acid, and the polybase, polyvinylamine, confirmed the fact that, while all the reagents used esterify carboxyl groups, only the epoxides react extensively with the amino groups (TABLE 2). Under our conditions no reaction with the phenolic group of tyrosine was observed.

Sulfhydryl groups. Since bovine serum albumin contains only one —SH group per molecule, reaction with this type of group was studied in ovalbumin, which has five times as many on a weight basis. In the native protein no reaction could be found. This was not unexpected since, in the undenatured state, the three groups per molecule are known to be inaccessible to other and more powerful —SH reagents. Denaturation revealed these masked —SH groups; after solutions of ovalbumin had been heated at 100° C. for 5 min. at *p*H 7, all three groups became available for reaction and were readily alkylated by the radiomimetics listed in TABLE 3. Earlier, Bacq[3] had demonstrated extensive reaction between sulfur mustard and the —SH groups of denatured proteins. All alkylating agents appear to react readily with the —SH groups of proteins although, in the native state, not all are accessible. Reasons for believing that this reaction is not important in the production of the radiomimetic biological effect are presented by W. C. J. Ross in the preceding paper.

TABLE 3
REACTION OF ALKYLATING AGENTS WITH THE —SH GROUP
Three Per Cent Egg Albumin Before and After Denaturation

Alkylating agent*	Per cent—SH groups reacted	
	Native protein	Denatured protein†
$(ClCH_2{\cdot}CH_2)_2N{\cdot}C_6H_4{\cdot}(CH_2)_3{\cdot}COOH$, chlorambucil (1.7% solution)	14	88
$CH_3{\cdot}SO_2{\cdot}O{\cdot}C_2H_5$ (2.6% solution)	0	55
 CH_2—$CH{\cdot}CH_3$ (5% solution)	0	100

* Reaction time 4 hr. at 37° C.
† Denatured by guanidine.

Amino acid analyses. Two-dimensional chromatography of the acid hydrolyzates of the treated proteins augmented the results obtained by analysis of the intact proteins. The substituted amines survived this treatment, although the esters formed by alkylation were destroyed by acid hydrolysis. The chromatograms of proteins treated with epoxides are characterized by the almost complete disappearance of the spot due to histidine (FIGURE 2) and a substantial reduction in the intensity of the lysine spot. None of the other amino acids seemed to have been affected, but certain new spots appeared.

With the bifunctional epoxides, the new spot was close to the origin, which suggested that it was due to two basic amino acids linked together through their side-chain amino or imino groups. With nitrogen mustards, similar new spots appeared (FIGURE 2) and the intensity of the histidine spot was reduced, although to a lesser extent. No change could be detected in the chromatograms of proteins treated with the mesyloxy compounds, confirming the findings shown in TABLE 1, that there was little or no reaction with the amino groups.

Discussion. These experiments have confirmed earlier evidence that the esterification of carboxyl groups is the one reaction with native proteins shared by all the alkylating agents. In the previous paper Ross has summarized reasons for believing it unlikely that reaction with amino groups contributes to the biological activity of the alkylating agents. These reasons are supported by the finding that, under our conditions, only the epoxides were outstanding in their reactivity with protein amino groups, while the other alkylating agents with equal or greater biological activity reacted only to a very small extent.

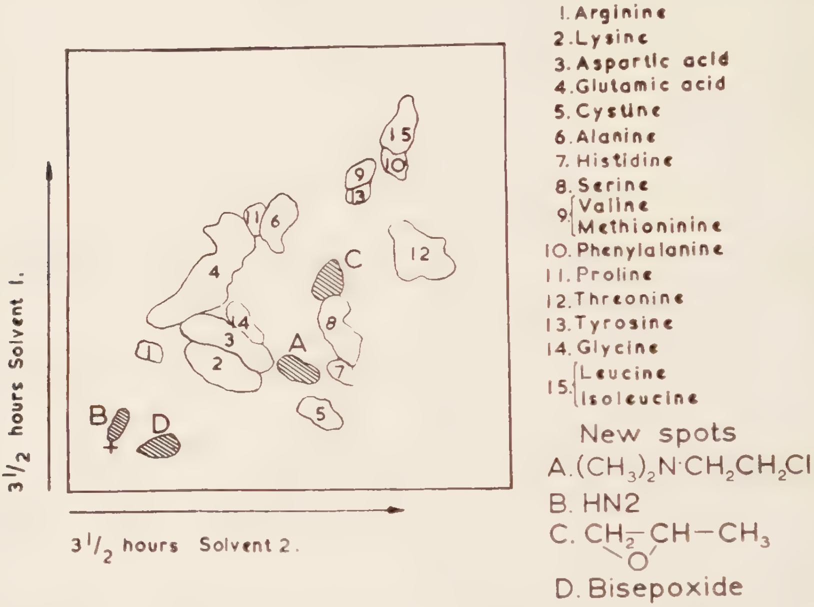


FIGURE 2. Two-dimensional chromatogram of hydrolyzate of serum albumin. Solvent 1 contains ethanol, butanol, water, and propionic acid. Solvent 2 contains acetone, butanol, water, and dicyclohexylamine. The new spots were seen after treatment with the following alkylating agents: (A) $(CH_3)_2N\cdot CH_2\cdot CH_2Cl$; (B) $CH_3\cdot N:(CH_2\cdot CH_2\cdot Cl)_2$; (C) $\underset{O}{CH_3\cdot CH \cdot CH_2}$; and (D) $(\underset{O}{CH_2 - CH}\cdot CH_2)_2O$.

Reactions with the Nucleic Acids

*Deoxyribonucleic acid.** Elmore *et al.*[6] found that mustard gas esterified the primary phosphate groups of both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). They also suggested that changes in the shape of the titration curve indicated some reaction with amino and sugar hydroxyl groups, but more recent work, notably by A. R. Peacocke, has made this interpretation unlikely. Alexander[7] showed subsequently that the phosphate groups of DNA could be esterified by all the radiomimetic alkylating agents.

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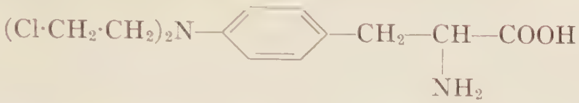
* The DNA used in these experiments was obtained by a modification of the procedure given by Kay *et al.*[4] from herring sperm heads.[5] For the various samples used in the experiments reported here, the molecular weight obtained by light scattering was slightly greater than 6 × 10^6.

Unambiguous evidence has now been obtained with the aromatic nitrogen mustard derived from phenylalanine (CB 3025),



that, when there is an excess of phosphate groups, as there is when the drug is used *in vivo*, reaction with DNA is confined entirely to esterification.

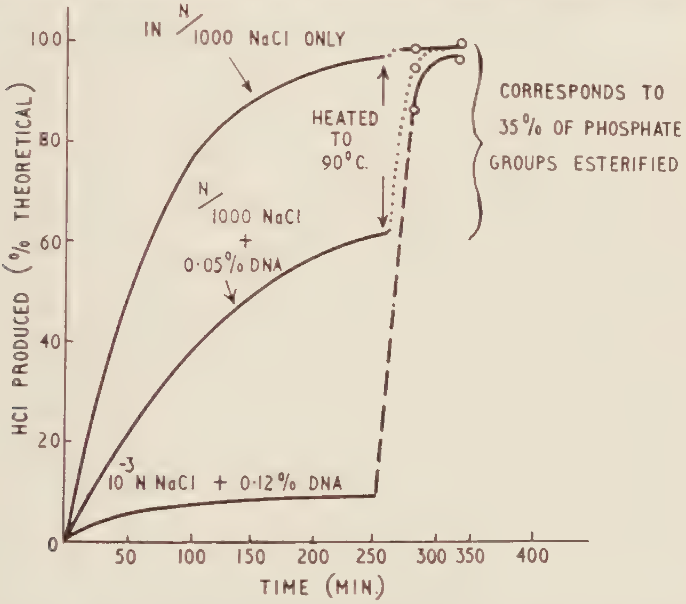


FIGURE 3. Rate of acid formation when the phenylalanine nitrogen mustard at a concentration of 0.1 per cent was reacted with different concentrations of DNA at 37° C. (*p*H was maintained at 7 by an automatic titrator), and the effect of subsequent heating for 20 min. at 90° C.

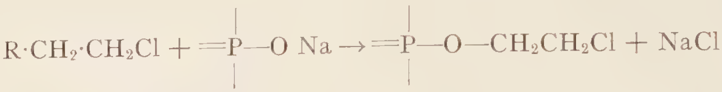
In the absence of any other reactive substance, aqueous solutions of the mustards hydrolyze with liberation of acid:

$$R{\cdot}CH_2{\cdot}CH_2Cl + H_2O \rightarrow R{\cdot}CH_2{\cdot}CH_2{\cdot}OH + HCl$$

The progress of this reaction can be followed at constant *p*H by using an automatic titrator, a "*p*H stat.," and noting the amount of alkali added at different times. Since first-order kinetics are obeyed, curves such as that shown in FIGURE 3 are obtained. In reaction with an amine, the same amount of acid is released as on hydrolysis,

$$RCH_2{\cdot}CH_2Cl + {-}NH_2 \rightarrow {-}NH{\cdot}CH_2{\cdot}CH_2R + HCl$$

but on reaction with an anion there is no liberation of acid,

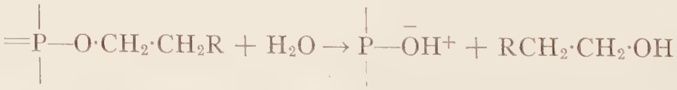


$$R\cdot CH_2\cdot CH_2Cl + =\overset{|}{\underset{|}{P}}\text{—O Na} \rightarrow =\overset{|}{\underset{|}{P}}\text{—O—}CH_2CH_2Cl + NaCl$$

The difference in the amount of acid released in the absence and presence of an anion is therefore a measure of the amount of mustard involved in esterification. In the experiment shown, one of the curves in FIGURE 3, 40 per cent of the mustard has reacted with the phosphate groups of DNA. Since the amount of acid released is not affected if reaction occurs with an amino group, it is not possible to estimate from the titration curve how much of the remaining 60 per cent was used in reactions with the amino groups of the nitrogen bases. This information was obtained by dialyzing the solution after reaction when any mustard that had hydrolyzed, but not reacted with the DNA, diffused through the cellophane. In this way it was found that, in these experiments, at least 90 per cent of the mustard not used up in esterification was lost by hydrolysis.

The same conclusion was reached by kinetic analyses of experiments with increasing concentrations of DNA. These suggest that less than 1 amino group reacts for every 10 phosphate groups. That this was the case in relatively concentrated solutions of DNA can be seen by inspection of the curve in FIGURE 3 for a 0.1-per cent solution of DNA. Here 94 per cent of the mustard reacted with phosphate groups. Of the remaining 6 per cent, it is likely that some was hydrolyzed so that in this case reaction with the amino groups must have been confined to less than one twentieth part of that used in esterification.

The alkylation of the phosphate groups of DNA resulted in the formation of triesters that, according to a suggestion of Brown and Todd,[8] should be unstable. This prediction was confirmed by heating, for 20 min. at 90° C., DNA that had been reacted with a nitrogen mustard. In this time an amount of acid exactly equal to the amount of esterification was released (FIGURE 3). Since no detectable amount of acid was produced by heating DNA that had not been treated with a mustard, it can be concluded that this acid is formed by hydrolysis of the triesters:



$$=\overset{|}{\underset{|}{P}}\text{—O}\cdot CH_2\cdot CH_2R + H_2O \rightarrow \overset{|}{\underset{|}{P}}\text{—}\overset{-}{O}H^+ + RCH_2\cdot CH_2\cdot OH$$

Dialysis of the solution after heating was used to separate the mustard, and the amount released by hydrolysis was estimated in a spectrophotometer. Within experimental error (±5 per cent), it appeared that fission of the triester occurred at the mustard link; no hydrolysis of the main chain could be detected although, by using a more sensitive method (TABLE 4), it was found that this reaction does occur, although very infrequently.

These hydrolysis experiments provide further support for the view that the L-phenylalanine mustard does not react with amino groups when DNA is present in excess. The alkyl bond with amino nitrogen is much more stable, so that any mustard combined in this way would not be affected during the 20-min. heating at 90° C., which causes complete fission of the triesters. Within experimental error, however, all the mustard that had been combined with DNA was released by this treatment.

The reaction of the epoxides with DNA cannot be followed in this way, since the reaction kinetics are second order and, therefore, affected by the presence of other solutes. No acid or alkali is released on hydrolysis or reaction with an amine:

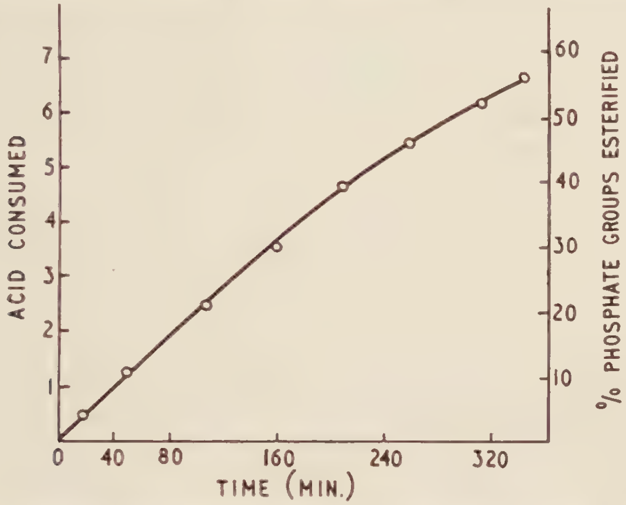
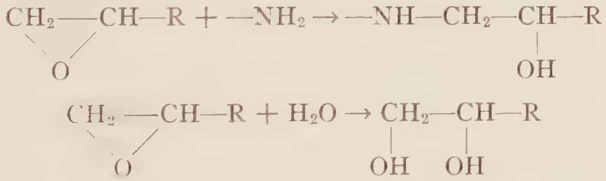
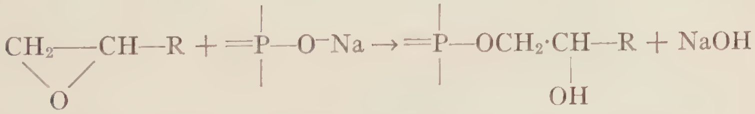


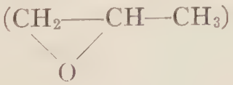
FIGURE 4. Rate of alkali produced (that is, acid consumed to maintain *p*H at 7) when 0.1 per cent DNA was treated with a 2.0 per cent solution of propylene oxide at 37° C.

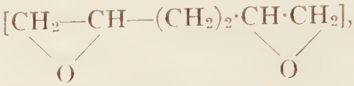


Reaction with an anion, however, produces alkali, so that the automatic titrator can be used to follow the course of the esterification reaction (FIGURE 4).



The rate of reaction of the two epoxides used, propylene oxide



and hexane bisepoxide  $[\underset{O}{CH_2-CH}-(CH_2)_2\cdot\underset{O}{CH\cdot CH_2}]$, is so much slower than that of the mustards that large concentrations of epoxide were used to obtain a convenient rate of reaction. Only a small fraction of the reagent was consumed during the time of the experiment, either by hydrolysis or by reaction with DNA.

As in the case of the mustards, the predominant reaction is one of esterification. Although, after long treatments (for example, when about 50 per cent of all the phosphate groups have been esterified) with excess epoxide, changes in the ultraviolet absorption spectrum are noticed. There is increased absorption at 270 mμ, and this suggests reaction with groups other than phosphate, such as the amino group of guanidine, or one of the ring nitrogens of the purines or pyrimidines.

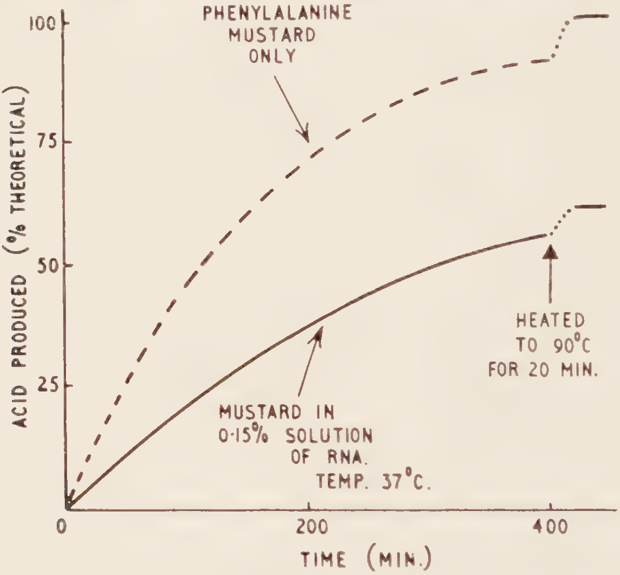


FIGURE 5. Rate of acid formation when 0.1 per cent phenylalanine mustard reacts with RNA.

Ribonucleic acid. Only preliminary experiments have been performed thus far with a sample of RNA obtained from rat liver by the method given by Kay *et al.*[1] Again, it appeared that reaction was confined to esterification of phosphates when this material was reacted with the L-phenylalanine mustard, since in 0.2-per cent RNA solutions the acid liberated was only 10 per cent of that released by hydrolysis (FIGURE 5).

There was, however, one remarkable difference between the two nucleic acids after reaction. When the esterified ribonucleic was heated, practically no acid was liberated. There is no obvious reason why the triesters of RNA should be more stable than those of DNA; possibly the stable esters in RNA were formed by reaction with secondary phosphate groups, of which there are very many more in RNA (because they occur as terminal groups) than in DNA, which has a far higher molecular weight. However, this reaction requires far more study before any conclusions can be advanced.

Deoxyribonucleoprotein. A most convenient source of a pure deoxyribonucleoprotein (DNP) that has retained its biological integrity is herring sperm that has plasmolyzed in water to release the sperm heads. These nuclei are spheres 2μ in diameter, containing only the basic protein, protamine (61 per cent), and DNA (39 per cent), surrounded by an extremely thin membrane.[9]

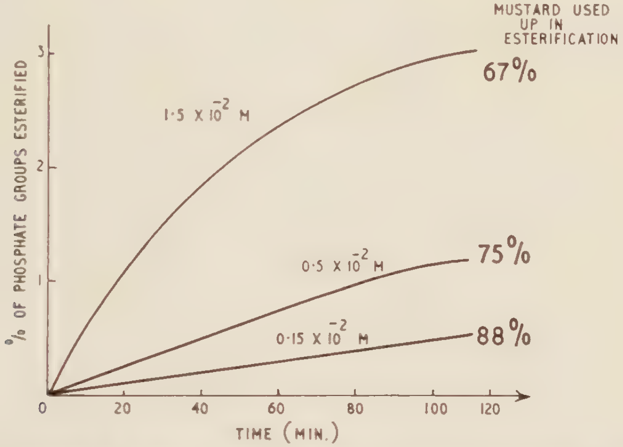


FIGURE 6. Reaction of three different concentrations of phenylalanine mustard with a 10 per cent suspension of herring sperm heads. The percentage of esterification was computed from the amount of acid liberated.

A suspension of these sperm heads reacted readily with all the radiomimetics. The esterification was followed in the same manner by determining the release of acid or alkali.

With small concentrations of the phenylalanine mustard, there was virtually complete suppression of acid liberation, showing that, essentially, esterification was the only reaction. With higher concentrations of mustard the acid liberated could be accounted for by hydrolysis, and the amount of mustard shown by dialysis to be combined with DNP was equal to the number of acid groups that had been esterified, as calculated from the reduction in acid liberated (FIGURE 6). The only carboxyl groups available for alkylation are those at the ends of the polypeptide chain and, at most, these could not have contributed more than 10 per cent to the reaction observed.

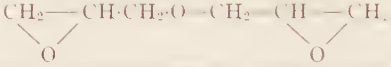
Degradation of Nucleic Acid by Main-Chain Fission During Hydrolysis

Although, as the results given above indicate, only a small fraction of the unstable triesters hydrolyze at one of the bonds in the main polynucleotide chain, a slow fall in the molecular weight of DNA (measured by light scat-

TABLE 4
MAIN-CHAIN HYDROLYSIS OF ALKYLATED DNA

Reagent	Per cent esterification	Time after reaction (hr.)	Temperature (° C.)	Molecular weight
CB 3025	~3	24	0	6×10^6
		24	37	2.25×10^6
	~6	24	0	6×10^6
		24	37	3.6×10^6
	26	24	0	2.7×10^6
		50	37	1.5×10^5
Propylene oxide	49	48	0	6.7×10^6
		24	37	3.3×10^6
Bisepoxypropyl ester	42	48	0	7.4×10^6
		24	37	2.15×10^6
HN2	20	24	0	5.4×10^6
		6	37	3×10^5

TABLE 5
REACTION OF A BISEPOXIDE WITH SODIUM POLYPHOSPHATE*

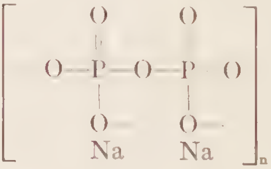


	Ratio $\frac{\text{epoxy group}}{\text{P O}^-}$	Molecular weight
One per cent polyphosphate	0	3.8×10^5
	⅕	2.0×10^5
	⅓	1.6×10^5
One-tenth per cent polyphosphate	0	3.8×10^5
	⅒	3.8×10^5
	⅕	3.4×10^5
	⅖	0.9×10^5

* Incubated at 37° C. at pH 7.5 in $M/60$ bicarbonate buffer. The control is stable under these conditions.

tering) is always observed (TABLE 4). Hydrolysis of one of the two chains of the twin helix does not necessarily cause a fall in the molecular weight; the organized structure may be held together by the other chain and thus conceal a single break.[10] The unmasking of these single breaks will be dealt with in more detail in our other contributions to this monograph.

The synthetic polyphosphate



$$\left[O{-}\overset{O}{\underset{ONa}{P}}{-}O{-}\overset{O}{\underset{ONa}{P}}{-}O \right]_n$$

having only one chain, demonstrates the effect of main-chain hydrolysis more clearly (TABLE 5). Even here, however, an extensive reaction is necessary before the effect is at all marked.

Crosslinking

Intermolecular and intramolecular crosslinking. A polyfunctional alkylating agent can react with two different nucleophilic centers to form a bridge between them or, by combining successively with a group with two reactive functions, such as a primary amino or a secondary phosphate group, can form a cyclic structure. It is only in these two respects that they differ from the monofunctional agents. The bridge can be built between two groups within the same macromolecule (intramolecular crosslinking) or between groups in two different molecules (intermolecular crosslinking), as shown in FIGURES 7a and 7b. These two types of crosslinking can have very different consequences and, therefore, can usually be distinguished readily by physical measurements, particularly with the flexible long-chain polymers, of which DNA is an example.

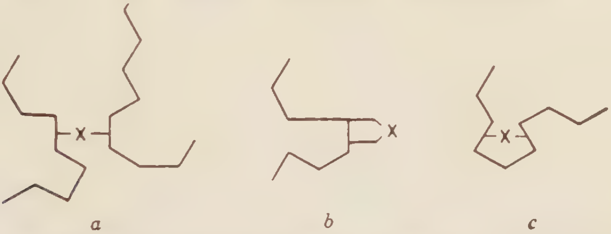


FIGURE 7. Different types of reactions produced by bifunctional reagents with a flexible macromolecule like DNA: (*a*) intermolecular crosslinking, (*b*) reaction with neighboring groups, and (*c*) intramolecular crosslinking.

Intramolecular crosslinking does not change the molecular weight but, if the bridge is between two different regions of the chain, it reduces the extent to which the polymer chain can take up more extended configurations and, therefore, it reduces the average value of its radius in solution. This in turn reduces the volume swept out by each molecule, an effect that can easily be observed as a reduction in viscosity. Crosslinking between nearby groups does not have this effect (FIGURE 7c).

Intermolecular crosslinking by joining two molecules together increases both the molecular weight and, for the flexible polymers, the viscosity. Crosslinking between two molecules obviously will occur more readily in relatively concentrated solutions of polymer, while intramolecular reaction is favored in dilute solutions. In practice, the change from one type to the other occurs over a narrow range of concentration. When a certain maximum number of molecules have been linked intermolecularly they form a gel, and the onset of gelation may be quite sharp.

The reaction of polymethacrylic acid with HN2 illustrates these differences in behavior very clearly. With the ratio of mustard to polymer maintained

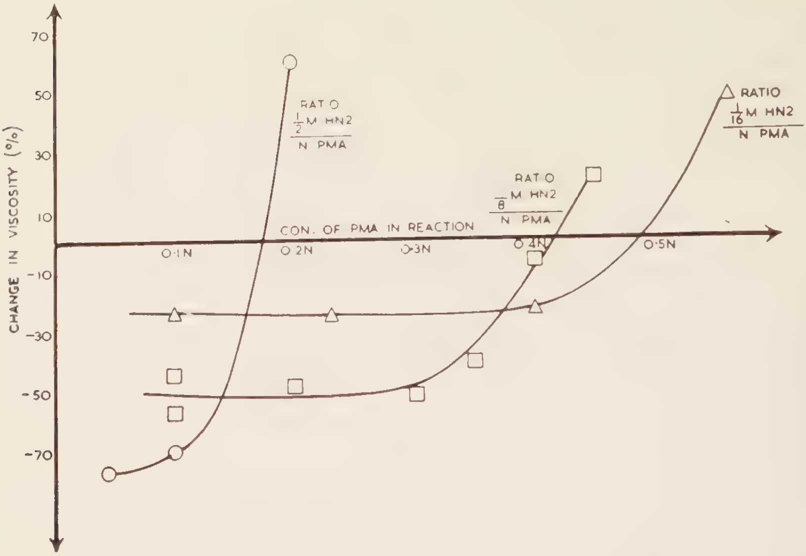
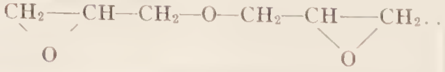


FIGURE 8. Change in the viscosity of solutions of 60 per cent neutralized polymethacrylic acid (PMA) after treatment with the mustard $CH_3N\cdot(CH_2CH_2Cl)_2$ (HN2). Each of the curves represents a series of experiments in which the ratio of polymer to mustard was constant, but in which the absolute concentration at which the reaction was carried out differed. Where the reaction was done at a low concentration the viscosity fell because the polymer molecule was crosslinked internally, whereas at high concentrations it rose because links had been produced between different molecules. The concentration at which the changeover between the two reactions occurred depends on the relative amount of mustard.

TABLE 6
CROSSLINKING OF BOVINE SERUM ALBUMIN[11]
Incubated at 40° C. for 5 Days at *p*H 7 in Phosphate Buffer

Reagent	Molecular weight	
	0.5 per cent protein solution	2.0 per cent protein solution
Control	74,000	74,000
HN2	72,000	170,000
$(CH_3)_2N\cdot CH_2CH_2Cl$	70,000	74,000
Myleran	74,000	78,000
 $CH_2{-}CH{-}CH_2{-}O{-}CH_2{-}CH{-}CH_2$	72,000	120,000

constant, the effect of the polymer concentration is shown in FIGURE 8 by plotting the change in the viscosity of the solutions after reaction as a function of the concentration. In dilute solution the viscosity falls in proportion to the mustard-to-polymer ratio due to the increased coiling by internal crosslinking. However, as the concentration of polymer is increased, there is a sudden change-over from internal to intermolecular crosslinking that causes the solution to set to a gel.

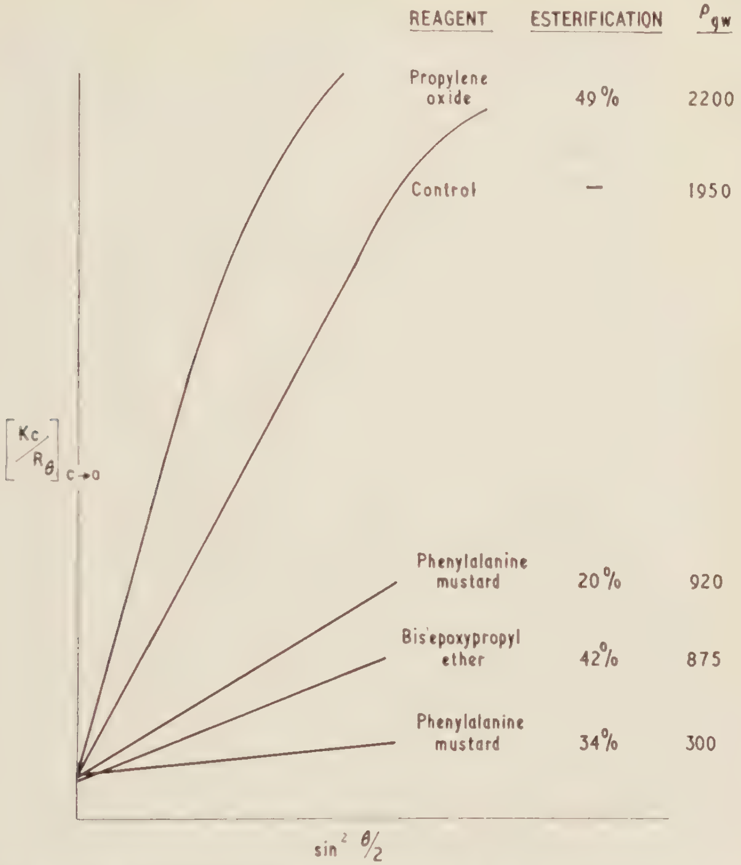


FIGURE 9. Light scattering spots (the reciprocal of the scattering at each angle is plotted as a function of that angle) for DNA treated with various alkylating agents. The radius of gyration, ρ_{gw}, obtained from the slope of the corresponding line, is a measurement of the extension in space of the DNA molecule after reaction. The molecular weight is inversely proportional to the intercept.

The shape of the relatively compact molecules of the globular proteins cannot be altered without denaturation, but intramolecular crosslinking in the more concentrated protein solutions can be detected reliably by the change in molecular weight (TABLE 6).[11]

DNA, although by no means as flexible as polymethacrylic acid, can be crosslinked internally by the bifunctional alkylating agents. This is clearly demonstrated by light-scattering measurements showing (FIGURE 9) that, immediately after reaction with these reagents, the molecular weight is unchanged, but the average volume of the molecules, expressed by the radius of gyration, is greatly reduced (TABLE 7). This "coiling up" is revealed clearly in the electron microscope. Comparison of DNA before and after treatment with phenylalanine mustard is shown in FIGURES 10 and 11. This decrease is responsible for the initial fall in the viscosity of DNA solutions observed to follow reaction with the mustards. The subsequent fall is due to the main-chain fracture referred to earlier.

TABLE 7
INITIAL EFFECT OF ALKYLATION ON THE SIZE OF DNA

Reagent	Per cent	Molecular weight	Radius of gyration
Control	—	6.4×10^6	2000
CB 3025	6	6.0×10^6	1100
	~20	5.4×10^6	980
	21	6.0×10^6	1080
	26	2.7×10^6	350
	34	8.0×10^5	220
	53	8.0×10^5	200
HN2	~10	6×10^6	1530
	~25	4.8×10^6	1040
CB 1348	0	5.8×10^6	1800
Propylene oxide	49	6.7×10^6	2200
Bisepoxypropyl ether	20	20×10^6	1350
	42	7.4×10^6	875

Crosslinking between molecules occurs at higher concentrations than we have used, and gels were produced by Elmore *et al.*,[6] reacting mustard gas in very concentrated solutions of DNA in ethanol. Such concentrations of nucleic acid are obtained in the sperm heads, and intermolecular crosslinking by all the radiomimetics has been observed. Dissociation of the unreacted nucleoprotein by 2 *M* NaCl gives a completely homogenous solution that is unchanged by centrifugation at 20,000 g for 1 hr. After reaction with a radiomimetic, which appears to involve only phosphate esterification, centrifugation discloses the presence of highly swollen gel particles by sedimenting them into a pellet. When the sperm heads used in the three experiments shown in FIGURE 6 were treated in this manner, 91, 83, and 16 per cent, respectively, of the DNA was present as gel that could not be dissociated by 2 *M* salt. This shows that crosslinking of DNA takes place very efficiently under these conditions.

The possibility that the gel is produced by crosslinking molecules of DNA and protamine was eliminated by measuring the protamine concentrations before and after centrifugation. There appeared to be no change in the

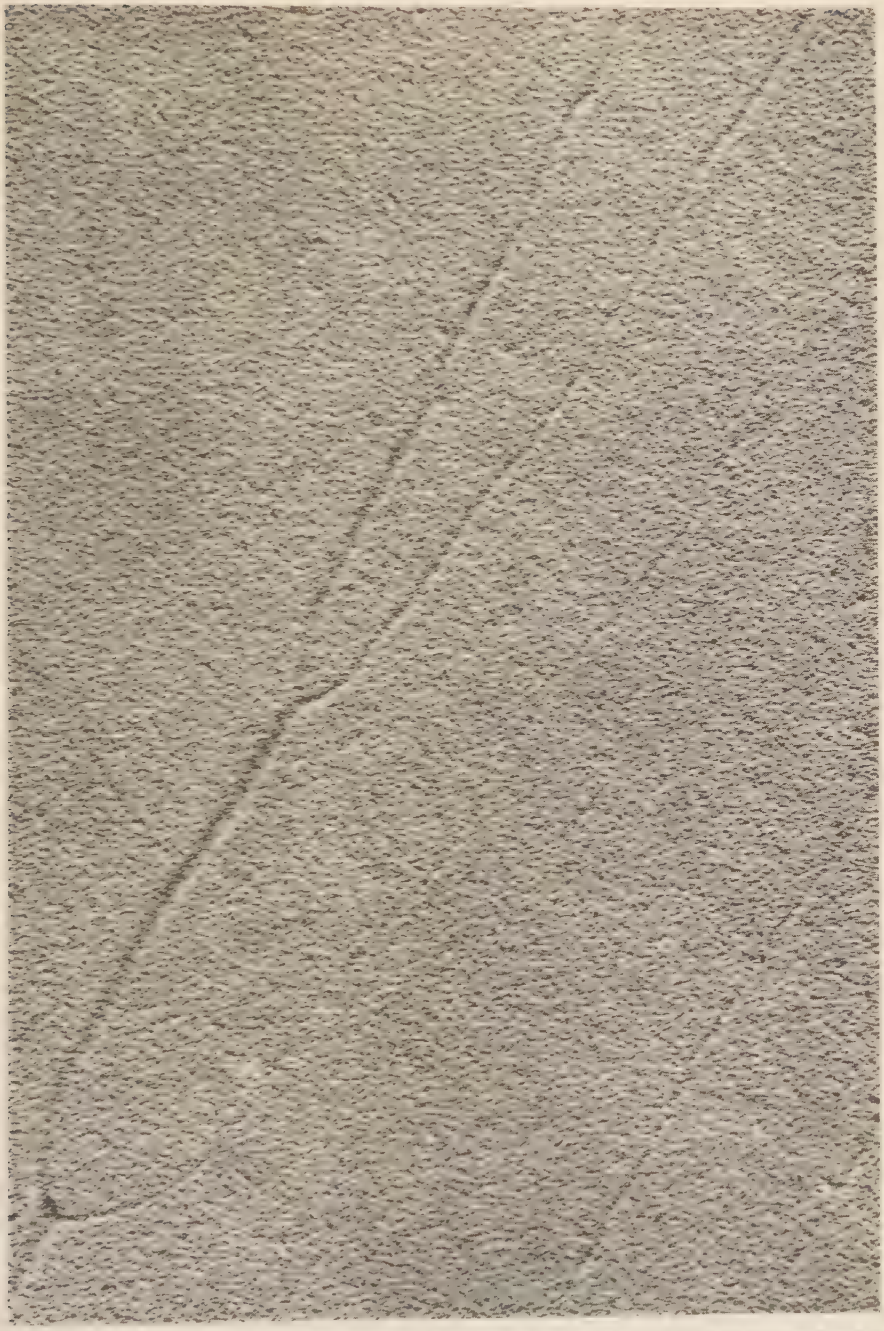


FIGURE 10. Electron micrograph of DNA which shows the tendency of two molecules to come together as a side-by side aggregate under the conditions used here. The thinnest threads are single molecules (that is, presumably double helices, since they are 20 Å. wide).

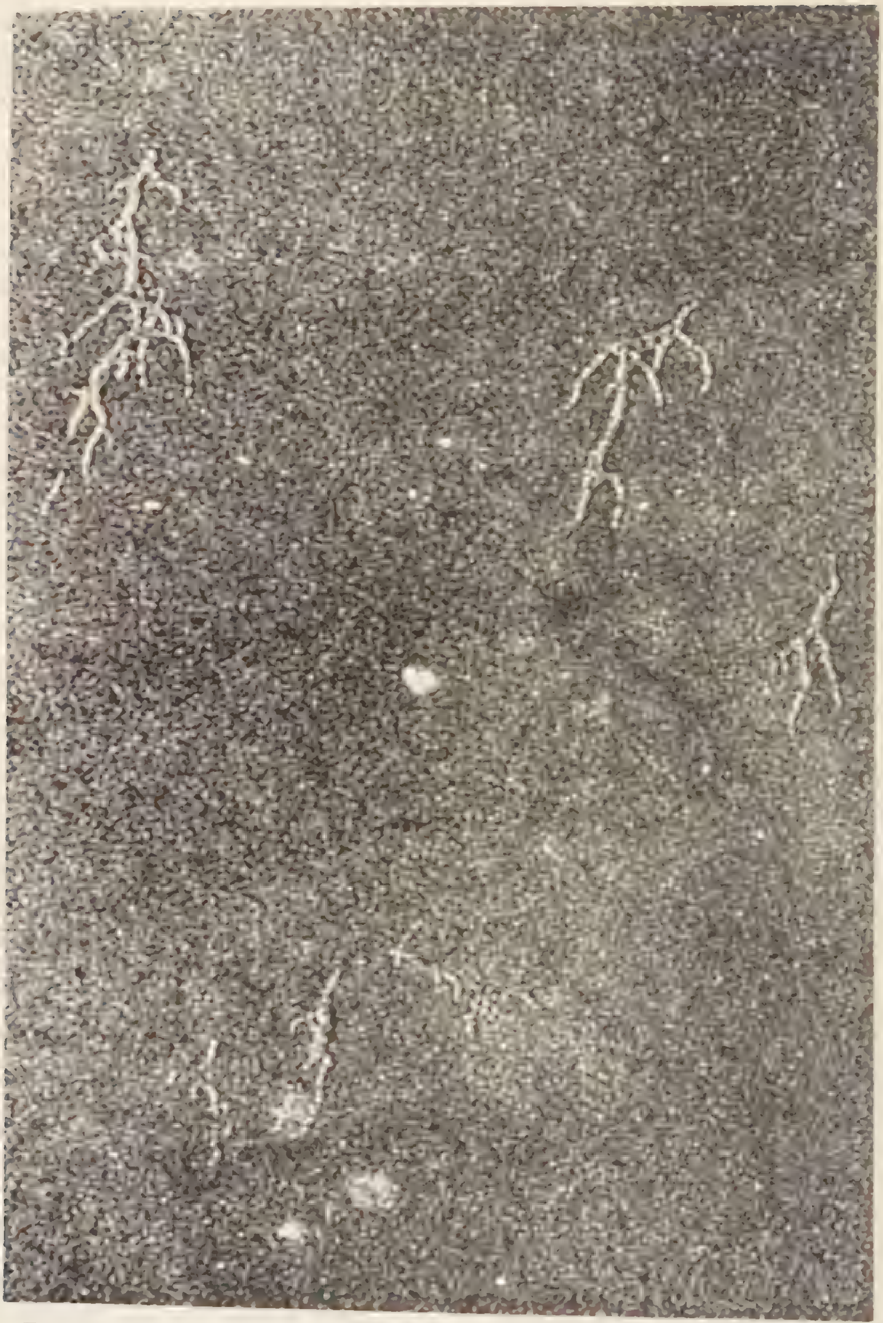


FIGURE 11. Electron micrograph of DNA after reaction with phenylalanine mustard. The cross links have prevented the straightening, and the enforced proximity of different points of the chain has facilitated the side-by-side aggregation.

protamine concentration of the supernatant, although as much as 90 per cent of the nucleic acid had been spun down, showing that the gel produced contained only nucleic acid.

Conclusions

Although the radiomimetic alkylating agents react with primary amino and sulfhydryl groups, our *in vitro* studies indicate that under physiological conditions, that is, when the number of sites available for alkylation far exceed the amount of reagent, esterification of an anion such as the carboxyl group in proteins and the phosphate groups in nucleic acids is the most characteristic reaction. The relative proportion of reaction with proteins and nucleic acids (or nucleoproteins) probably varies from one compound to another. The mere formation of these esters is not a very effective method of inducing radiomimetic effects, since monofunctional compounds have, in general, much less biological activity than those containing two or more alkylating centers per molecule. Similarly, the rare event of hydrolytic fission of the triesters formed with nucleic acid phosphate groups causing a break in the main chain seems unlikely to be of biological importance, because this, too, can be brought about by monofunctional alkylating agents. The importance of this reaction will be discussed in more detail, however, in our other paper elsewhere in this monograph (p. 1225). It seems very probable that it is their polyfunctional character that enables them to produce cross links either between different groups in the same molecule or between molecules, which is the outstanding characteristic of the highly active compounds. Either of these two forms of crosslinking will completely alter the character of a macromolecule and would be expected to prevent it from exercising its normal biological function. The inactivation of transforming principle that Zamenhof *et al.*[12] found to take place very readily on treatment with bifunctional nitrogen mustard can probably be ascribed to internal crosslinking.

In cells where the DNA nucleoprotein is present in high local concentration, crosslinking between molecules leading to the formation of gels will probably be favored. It is interesting that the phosphate groups in herring sperm nucleoprotein are so readily available for reaction, since the positively charged groups of the protamines are believed to be particularly and intimately associated with the phosphate groups of the DNA. There is, therefore, no reason to suspect that this reaction would not occur equally well with the DNA proteins in the nucleus. Preliminary experiments on the reaction of whole cells support this view, and it is our intention to study the reaction of the alkylating agents with the different morphologic components of the cell.

The data on RNA are too limited to permit any deductions concerning the possible effects of the reaction of the alkylating agents with RNA protein. The only relevant data would appear to be those of Herriott,[13] who records that tobacco mosaic virus is relatively much less insensitive to treatment with mustard gas than some DNA-containing viruses, but that the number of groups necessary for inactivation could not be measured in this experiment. Wood *et al.*[14] treated this virus with a one-armed sulfur mustard and found that, even after 1500 residues of the alkylating agent had combined, the virus had not lost any biological activity. To reduce the activity of the virus by one half, it was necessary to alkylate an average of 3200 groups per virus particle. These authors found that the one-armed mustard reacted equally with the protein (95 per cent) and with the RNA (5 per cent) moiety of the virus; consequently, one can deduce that as many as 75 mustard groups can combine with each RNA molecule without affecting its biological activity. It would be most valuable to know whether this remarkable resistance is due to the fact that a monofunctional agent was used or whether RNA is inherently affected less easily by alkylation than is DNA.

Summary

The reaction of representative nitrogen mustards, epoxides, and esters of methanesulfonic acid with proteins, DNA, and nucleoprotein have been studied.

(1) In serum albumin the predominant reaction occurred with carboxyl groups: these were readily esterified by all the reagents studied. The epoxides also alkylated the amino and imidazole groups, but other substances such as Myleran, HN2, and chlorambucil reacted much less readily with these groups. Experiments with synthetic polymers containing either only carboxyl groups or only amino groups confirmed the esterification of the carboxyl group as the predominant reaction.

(2) With DNA, by far the most reactive center is the primary phosphate group, and under conditions where DNA was in excess (as would be the case *in vivo*) it could be shown for the mustards that only esterification of the phosphate group occurred.

(3) With synthetic polymers, proteins, and DNA the bifunctional reagents produced cross links within the same macromolecule if these were reacted in dilute solutions (that is, of less than 1 per cent). At higher concentrations the bifunctional reagents crosslinked different molecules and, under favorable conditions, the whole solution then set to a gel. Internal crosslinking leads to a greater coiling of the DNA and is responsible for the drop in viscosity that has been observed by many workers.

(4) Immediately after reaction, the molecular weight of DNA is not reduced, but it falls on standing at 37° C. because the triphosphate esters produced by the alkylating agents are unstable and hydrolyze. For about 90 per cent of the triesters, the group removed is that introduced by the alkylating agent. However, hydrolysis of the main nucleotide chain also occurs at a very slow rate. This brings with it a decrease in molecular weight but is unlikely to be important physiologically. We believe the crosslinking reactions to be more significant, especially since, in recent experiments, they have been shown to occur when intact nucleoproteins (sperm heads) are exposed to nitrogen mustard. The reaction here was entirely with the phosphate groups of DNA, and none could be detected with the protamine.

References

1. Alexander, P. 1954. Advances in Cancer Research. **2**: 2.
2. Haddow, A. 1953. Biological alkylating agents. *In* The Physiopathology of Cancer. F. Homburger and W. H. Fishman, Eds. Hoeber. New York, N. Y.
3. Bacq, Z. M. 1946. Bull. acad. roy. méd. Belg. **11**: 137.

4a. Kay, E. R. H., N. S. Simmons & A. L. Dounce. 1952. J. Am. Chem. Soc. **74**: 1724.
4b. Kay, E. R. H. & A. L. Dounce. 1952. J. Am. Chem. Soc. **75**: 4041.

5. Alexander, P. & K. A. Stacey. 1955. Biochem. J. **60**: 194.
6. Elmore, D. T., J. M. Gulland, D. O. Jordan & H. F. Taylor. 1948. Biochem. J. **42**: 308.
7. Alexander, P. 1952. Nature. **169**: 226.
8. Brown, D. M. & A. R. Todd. 1952. J. Chem. Soc. : 52.
9. Felix, K., H. Fischer & A. Krekels. 1956. Progr. in Biophys. and Biophys. Chem. **6**: 1.
10. Alexander, P. & K. A. Stacey. 1955. Progress in Radiobiology. : 107. Oliver and Boyd. Edinburgh, Scotland.
11. Alexander, P., M. Fox, K. A. Stacey & L. F. Smith. 1952. Biochem. J. **52**: 177.
12. Zamenhof, S., G. Leidy, E. Hahn & H. F. Alexander. 1956. J. Bacteriol. **72**: 1.
13. Herriott, R. H. 1948. J. Gen. Physiol. **32**: 221.
14. Wood, J. L., J. R. Buchele, C. H. Stevens, F. H. Carpenter & V. du Vigneaud. J. Am. Chem. Soc. **70**: 2547.

COMPARISONS OF THE BIOLOGICAL EFFECTS OF IONIZING RADIATIONS AND CERTAIN ALKYLATING AGENTS IN CELL SYSTEMS*

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In a discussion of the relative biological effectiveness of radiation and the alkylating agents, it is necessary to consider most carefully the large number of variables present in such systems. Biological test objects vary in response, not only according to the species and strain, but also according to many environmental factors, internal and external. In applying radiation, we must consider the kind of radiation, the total dose, the rate, the wave length, the presence or absence of other agents, and the aftertreatment. If the treatment is chemical, we must consider the molecular species of the test reagent and its concentration at the time and duration of the test; this is particularly important for many of the alkylating agents because of their rapid hydrolysis at physiological hydrogen-ion concentrations. In addition, the presence of other competing substances may change the dosimetry of the system.

In radiation experiments, control of the variables has reached such a stage that dosimetry has been almost eliminated as a problem. Even in large and complex organisms, knowledge of energy distributions is relatively good, so that comparisons of effects from organism to organism and from laboratory to laboratory are becoming reliable. However, when treatments with chemicals, especially alkylating agents, are compared, the situation is not as favorable. Not only are we uncertain about the nature of the various molecular species present in the solution, but we are even less certain about their distributions within the organism. As several investigators have pointed out (Powers *et al.*,[1] Visconti di Modrone[2]), there are many variables, even in cellular systems, that make it difficult to compare different kinds of treatment. However, experimental systems consisting of cultures of single cells are much simpler to work with than are more complex organisms; in addition, when testing with alkylating agents, it is easier to control the nature of the molecular species, its concentration, and the length of exposure. In this paper, we shall review the existing literature, discussing those papers that permit the comparison of the effects of the alkylating agents with ionizing radiation.

Chilomonas

The response of the colorless flagellate, *Chilomonas paramecium*, to nitrogen mustard has been described in detail by Hutchens and Podolsky.[3] Using direct counts of cells, these investigators have established (FIGURE 1) the

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* The work reported in this paper was performed under the auspices of the United States Atomic Energy Commission, Washington, D. C.

fact that in low concentrations of HN2* a culture in exponential growth will show some increase in numbers ($\sim$50 per cent) before a temporary suppression of the fission rate takes place. The magnitude of this suppression is proportional to the concentration of HN2 in the solution. After a period of block, there is a period of recovery. For a brief time the observed fission rate exceeds that of the controls and then, finally, the culture grows at the same rate as the controls. Concentrations of HN2 used were just below the values causing cell death. It is important to remember that the cells apparently become resistant to the nitrogen mustard, which is still present throughout the period of observation. When the cells are washed free of the mustard, they lose this resistance during the next fission.

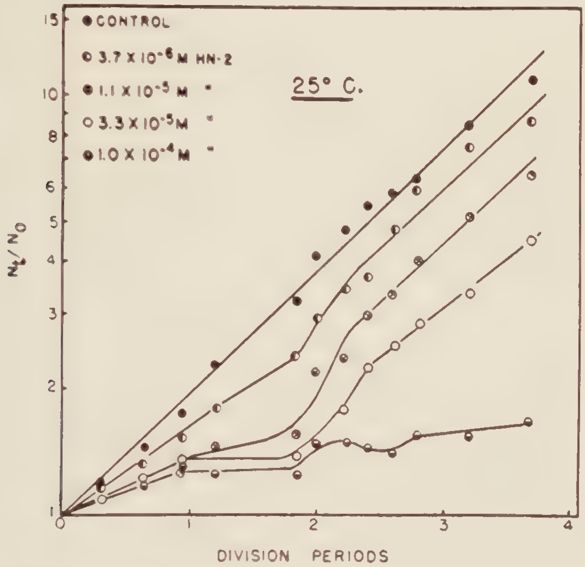

FIGURE 1. Growth of *Chilomonas paramecium* in HN2 solutions.[3]

The cells that are unable to undergo fission in the presence of the mustard do, however, continue to synthesize nucleic acids and proteins; that is, nitrogen mustards can stop fission effectively without interfering with certain important synthetic activities of the cell (Podolsky and Hutchens[4]). This observation is of considerable interest to those who wish to relate nucleic acid and protein synthesis with cell division. Podolsky and Hutchens suggest that the HN2 enters into a reversible combination with some cell components necessary for cell division. When these components are inactivated by combination with HN2, more is synthesized; the level of cell components governing division becomes higher, permitting division.

There are a number of observations of the effects of X rays on *Chilomonas* that allow direct comparison of X rays and alkylating agents. Ducoff,[5,6] using single dosages of X rays in the 5- to 20-kr range, showed that delay is immediate; that is, no division is observed in the log-phase cultures after X irradiation (FIGURE 2). This is different from the results seen after mustard treatment in *Chilomonas*. The survivors remain in fission block, the length of which is proportional to the dosage. Then recovery occurs, and fission rates return to the values of the control. As demonstrated for mustards, growth measured by increase in starch continues during fission block. Since no new cells are formed during the period of block, the existing cells grow larger.

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* 2,2′-Dichloro-*N*-methyldiethylamine hydrochloride.

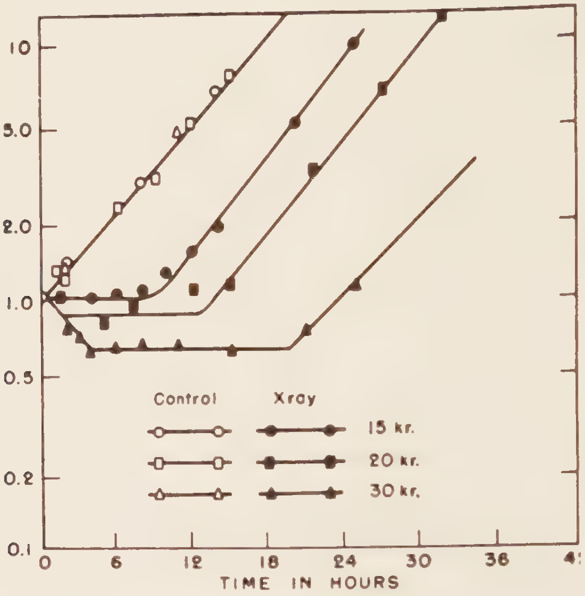

FIGURE 2. Growth of *C. paramecium* after treatment with X rays.[5, 6]

At dosages of either agent sufficient to cause death of some cells, fission is inhibited. However, the fission block, after either treatment, is reversible, and synthesis in the cells continues. X-ray treatment always causes immediate block, but HN2 does not cell multiplication may continue before complete inhibition. Cells recovering from HN2 treatment divide more rapidly for a brief period than do the controls, but cells treated with X rays do not show this unexpected acceleration during recovery. If the cells are kept in HN2, the inhibition is only temporary, and they resume control behavior. Nothing like this has been observed for cells exposed to ionizing radiation, although the observations of Rubin[7] on *Escherichia coli* are relevant.

Pelomyxa

Pelomyxa is a large multinucleate amoeba used recently by Daniels[8, 9] for the direct comparison of the cellular effects of HN2 and X rays. After exposure to low concentrations (5×10^{-5} to 2×10^{-4} molar) of HN2 for one hour during interphase, well-fed specimens of *Pelomyxa carolinensis* demonstrate very little delay in the first division. The second interdivisional period is very long; then there is a period of recovery, during which the treated cells divide more rapidly than do the controls. This is followed by a return to a normal growth rate (FIGURE 3). This pattern of delay and recovery is very much like that seen in *Chilomonas*, as noted above.

When young, unfed specimens of *Pelomyxa* are exposed to HN2, most of the cells are immediately blocked before their first division. On the other hand, when *Pelomyxa* is exposed to X rays, the period of inhibition, unlike that found in starved specimens treated with nitrogen mustard, begins almost immediately. There is no induction period corresponding to that observed in well-fed animals.

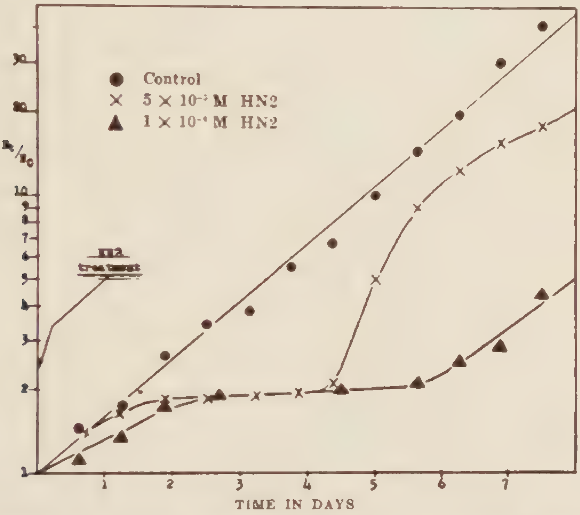

FIGURE 3. Behavior of *Pelomyxa carolinensis* after treatment with X rays and HN2.[8, 9]

Daniels further showed that untreated protoplasm acts as a therapeutic agent, reversing the effect of X rays or mustards. As may be seen in FIGURE 4, halves of animals that have been treated with either X rays or mustards can be fused with untreated halves; in either instance, the composite animals then have nearly the same growth rate as do the controls. If the mechanisms of action, or the sites of damage, of these two treatments were different, it might be possible to use X-irradiated protoplasm to reverse the effects of HN2, or vice versa. Daniels tried these experiments but, as this figure shows, no therapeutic effects were observed. These results may be interpreted as showing that the two agents operate in the same protoplasmic systems to inhibit cell division.

We should note here that not all species of *Pelomyxa* show the same characteristics; *P. illinoisensis*[10] treated with X rays usually undergoes one cell division before inhibition begins, but *P. carolinensis* becomes inhibited immediately. The latter animal, however, when treated with HN2, becomes inhibited after one division. No HN2 treatments of *P. illinoisensis* have been made. The fact that *P. illinoisensis* is much more sensitive to radiation than is *P. carolinensis* may be pertinent.

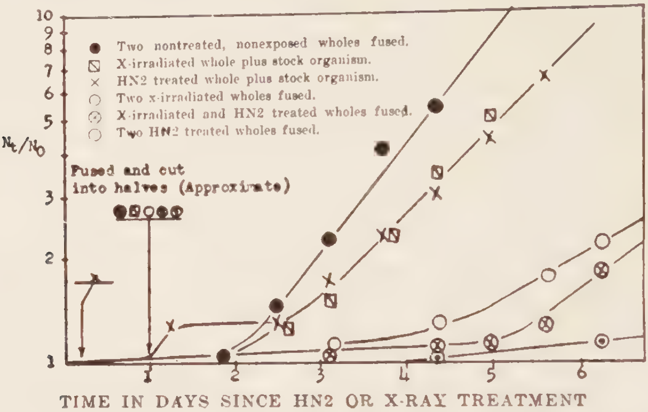

FIGURE 4. Behavior of *Pelomyxa carolinensis* after treatment with X rays and HN2.[8, 9]

Escherichia coli

In the last few years a number of studies of the effects of these agents on *E. coli* have appeared. Loveless *et al.*,[11] testing a large number of chemical compounds on *E. coli* B, demonstrated that cell division could be specifically inhibited without interfering with cell growth. Effective compounds included HN2, TEM (triethylene melamine), acriflavine, 5-diazouracil, diethylsulfate, dimethylsulfate, ethylenimine, 5-nitro-7-hydroxybenzotriazole, and β-propiolactone. The same authors reported different results in yeast; only HN2 and TEM allowed cell growth, as measured by increase in dry weight, during blocking of cell division.

Katchman[12] has contributed evidence pertinent to this matter. He found that *E. coli* was inhibited in its division rate by 5-diazouracil and also by TEM; growth continued at an exponential rate, but division stopped. The values of inorganic phosphorus, lipid phosphorus, and RNA and DNA phosphorus continued to increase at nearly the same rate as that of the controls for the 1½-hr. period of observation. This is another important example of an organism in which cessation of cell division does not appear to be a result of interference with RNA and DNA synthesis.

Corresponding experiments with radiation deal with similar capacities of the cells. Spoerl *et al.*[13] irradiated *E. coli* B continuously with X rays with total dosages up to 24 kr. Although division ceased and chain formation was prominent, growth, as the authors defined it, continued. Cells became four times as large as the controls, but the total dry weight of the cultures remained within 20 per cent of the control values. This indicates that protein synthesis probably still continued during the period of constant irradiation.

Rubin[7] has reported relevant results in *E. coli* B and B/r. Continuous irradiation of growing cultures was effected by adding P^{32} to the media. When an actively growing culture of *E. coli* B was added to a medium containing P^{32} (FIGURE 5) there was a short period of continued growth, followed within 1 to 2 hr. by a lag; this lag was not observed in other studies[11-13] that, however, were not carried on for this length of time. After a period depending upon the concentration of P^{32}, there was a resumption of growth, measured turbidimetrically (measured also by direct cell count under continuous X irradiation). This result appears to be similar to that in *Chilomonas*[3] exposed continuously to HN2 solutions, as well as in yeast exposed to sulfur mustards (Kinsey and Grant[14-16]). The general result was that continuous dosages of radiations caused complete fission suppression in growing cultures of *E. coli* B and B/r, and that the duration of the period of suppression was a function of the dose rate to which the cultures had been exposed. Further, nucleic acid synthesis continued at control rates in cultures receiving up to 5000 r/hr. for 10 hr.

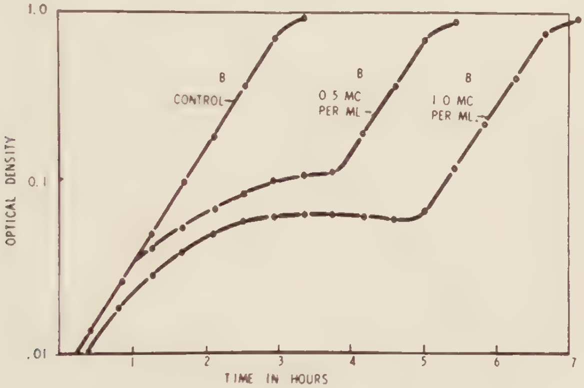

FIGURE 5. Growth of *E. coli* in P^{32} solutions.[7]

We note that specific inhibition of cell division in *E. coli* is brought about by many chemical agents, and that during fission block, synthesis in the cell continues at control rates. Continuous radiation also results in inhibition of cell division, while synthesis of nucleic acid continues. *E. coli*, like *Chilomonas* under continuous exposure to HN2, can resume cell division in the presence of the radiation source that caused initial suppression of fission.

Yeast

A number of investigators have attempted to analyze the actions of alkylating agents on yeast. Kinsey and Grant[14-16] showed that growth continued for some time after exposure to sulfur mustard. The culture was then inhibited, recovered, and then attained the division rate of the controls (FIGURE 6) in a manner similar to that with *Chilomonas*. The similarity, according to Hutchens and Podolsky,[3] is superficial, for in treated yeast cultures many cells die after a few divisions; secondary growth stems from the few survivors.

Other investigators have made studies of both chemical inhibitors and X rays on yeast cultures. Loveless *et al.*[11] tested a large number of chemical compounds for their capacity to inhibit fission. Of these, only HN2 and TEM inhibited cell division in what these authors called a specific manner, that is, associated with a continuing increase in size and dry weight. Loveless and his associates do not believe that yeast cells divide after exposure to HN2 and TEM.

The same workers[13] exposed yeast cultures to continuous X rays, mimicking the exposure of the cells to chemical agents. They reported that cell division was inhibited almost immediately, but there was no inhibition of increase in dry weight during the early period. This agrees with the results of Brace,[17] who demonstrated doubling of cell weight after 20 kr X rays at high dose rate.

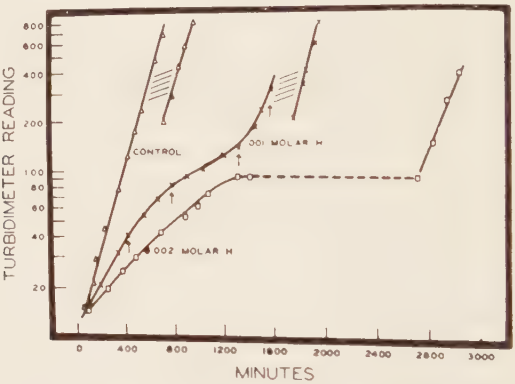

FIGURE 6. Growth of yeast cultures following treatment with sulfur mustard.[14]

According to another report from the same group (Spoerl and Carleton[18]), similar results were obtained after use of high concentrations of TEM or X rays, which reduced the cell count to 35 per cent of control and inhibited division. However, the individual cells became large, more than 2.5 times the size of the untreated cells.

Podolsky and Hutchens[4] observed yeast cultures for long periods after treatment (up to 8 hr.). In these cultures, in which several cell divisions were seen after treatment, concentration of nucleic acid and of protein was decreased at 2 hr. and later. The differences in time of the observations in the various experiments should be noted, for they may explain the apparent differences between them.

There are other radiation studies on yeast but, unfortunately, no chemical experiments of equivalent precision with which these can be compared. Burns[19] irradiated mature, diploid cells (1 to 50 kr) and observed that radiation was always followed by a short intermitotic time and then a very much longer intermitotic time, followed in turn by a shorter one. There was no correlation between the first short and the second long delay, indicating independence of these two effects. Division delay was not easily related to the ploidy of the cells, and so appeared to be a phenomenon independent of the nuclear conditions. This is in contrast to the lethality studies of Mortimer.[20]

In summary, it seems that the chemical inhibitors, as well as X rays, cause blockage of cell division, while allowing continued protein synthesis during the early period after treatment. There is evidence that at longer times after treatment profound depression of nucleic acid synthesis, followed by depression of protein synthesis, can be seen.

Studies of the lethality of X rays to yeast cells have been very fruitful contributions to cellular radiobiology, particularly the papers of Mortimer,[20] Wood,[21] and Zirkle and Tobias;[22] however, there are no corresponding publications on the effects of alkylating agents.

Amoeba

Other direct attempts to compare the actions of HN2 and X rays in inhibiting cell division and causing cell death have been described in a recent series of papers by Ord, Danielli, and others.[23-27] Without attempting to refer specifically to the individual papers, we present the general results.

Treated or untreated nuclei of *Amoeba proteus* can be transferred to treated or untreated cytoplasm of the same species. This makes it possible to analyze the sites where the agents are active, and to recognize the relative importance of damage to these sites. The desired concentration of mustard was used by these authors within 2 to 3 min. of the time of preparation for a standard exposure time of 15 min. As Powers *et al.*[1] have shown, the use of the mustard solution shortly after dissolving might cause difficulties, because the very active parent amine compound rapidly changes into the less active imine compound. When nuclei treated with HN2 were transferred into normal cytoplasm, many of the cells divided once or twice, perhaps more often (depending upon concentration), before death. This characteristic result indicated that the untreated cytoplasm was capable of carrying the cell through at least one division before the lethal damage in the nucleus was expressed. When a normal nucleus was put into treated cytoplasm, a different result was observed. First, none of these lethally affected cells divided before death. Second, lethality was brought about only by concentrations of mustard tenfold greater than those necessary for equivalent effects in treated nuclei. The LD_{50} for nuclei was 10^{-5} M HN2, whereas for cytoplasm it was about 10^{-4} M HN2. In other words, when the cytoplasm was lethally affected, no divisions took place. Also, the nucleus seemed to be about 10 times more sensitive than the cytoplasm. We should note, however, that on a unit weight basis, the cytoplasm, with its far larger volume, is probably much more sensitive than the nucleus.

Low concentrations of HN2 caused the nucleus of *Amoeba* to undergo a short delay before the first fission, followed by a much longer delay before the second fission.

The same techniques were used in a study of the sensitivity of these cells to single dosages of up to 300 kr of X rays. As with HN2, both cytoplasm and, perhaps, the nucleus could be directly damaged by the radiations. Irradiated nuclei transferred into unirradiated cytoplasm yielded results similar to those found in irradiated whole amoeba—a long delay occurred in the first division and a slight delay could occur in the second.

When normal nuclei were transferred into irradiated cytoplasm, the response depended very much on the time after irradiation of the transfer. If the transfer took place 1 to 2 days after irradiation of the cytoplasm, almost 100 per cent of the amoebae receiving 250,000 r survived the treatment, indicating that damage to the cytoplasm is reversible. In these, a delay usually occurred before the first division. At higher dosages to the cytoplasm, the damage might be irreversible. Nuclei appeared to be about 2½ times more sensitive to X rays for the induction of irreversible damage to the cell.

The patterns of division after X irradiation were not similar to those seen after HN2; if death was to occur, it would take place before the first division. This was in contrast to the results with mustards, in which damaged nuclei allowed several divisions of the cells before death occurred. When a delay in division was seen, all patterns of irradiation resulted in the highest delay in the first division. The second division was sometimes delayed when the cytoplasm was irradiated, but irradiation of the nucleus infrequently caused delay of the second division.

The authors concluded from the dose-response curves that a dosage of X rays lethal to all nuclei would not cause appreciable cytoplasmic damage, while the induction of a lethal effect in nuclei by nitrogen mustards would certainly cause some lethal cytoplasmic damage. These findings are comparable to those in *Paramecium* discussed by Powers and Ehret.[28]

Paramecium

Paramecium aurelia has been used by two groups of investigators to compare the effects of nitrogen mustards and X rays. The experimental conditions, however, were not strictly comparable: (1) different stocks and mating types of *P. aurelia* were used, and (2) Kimball *et al.*[29] used well-fed animals shortly after fission, while Powers *et al.*[1, 28, 30, 31] used animals that had been starved for from 12 to 18 hr. prior to exposure. The importance of the difference in stocks of the organism is not known; however, in the light of other experiments, we believe that the nutritional condition of these cells was significant, and that it explains most of the observed differences in response.

Kimball *et al.*[29] exposed well-fed *P. aurelia*, variety 1 (personal communication on the variety of *aurelia* used), to increasing dosages of X rays. This caused an inhibition of division, followed by a return to higher fission rates, followed in turn, at the third and fourth division, by a lowering of fission rate. A gradual return to control rates then occurred over the next 4 or 5 fissions. If we explain the first inhibition as being a result of the transfer of the irradiated organisms to fresh culture solution, then the general pattern was a depression of fission rate for 2 to 3 divisions, then gradual return to control rates over the next 4 to 5 divisions.

When the same authors exposed *P. aurelia* to nitrogen mustards, the same general type of inhibition was demonstrated maximum inhibition was observed at the third or fourth fission after treatment. The observation of cell death at the dosages of mustard at which fission inhibition occurred was in general agreement with the observations of many others.

Starved specimens of variety 4, *Paramecium aurelia*, suspended in a buffered salt solution containing no phosphate, were routinely used in our laboratory. Under these conditions, the results obtained by treatment with either X rays or nitrogen mustards were highly reproducible. Following treatment with HN2, there was an immediate long delay in fission, followed by a gradual recovery extending over a number of cell generations (FIGURE 7).

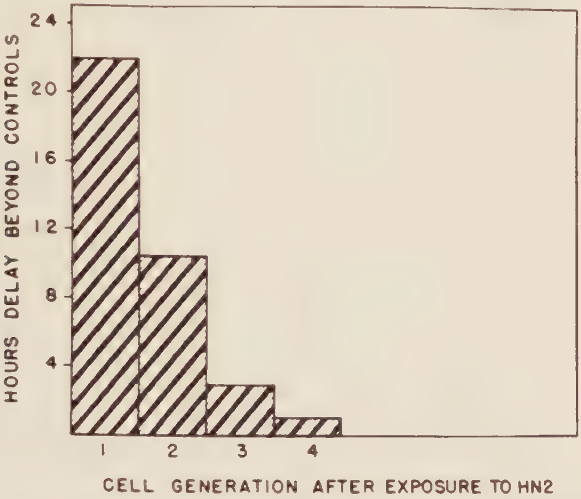

FIGURE 7. Changes in rate of cell division in *Paramecium aurelia* after treatment with HN2 (0.002 *M* for 1 hr.).[33]

One other effect is measurable, the induction of death after autogamy that presumably measures micronuclear damage in these organisms. Geckler[32] has measured a presumed micronuclear damage by nitrogen mustard in variety 4, *P. aurelia*, and discusses the difficulties in the assumption. His results, except for the quantitative relationships, are similar to those we observed in our laboratory with our sublines of the same stock. The general relationship to increased dose (FIGURE 8) was that death after autogamy increased as the time of exposure increased, falling after a peak response was reached. The general relationships between these effects, as well as the incidence of death before cell division, is shown in FIGURE 9, which demonstrates that, after nitrogen mustard treatment, three effects were observed at about the same concentration. The general features of the response to

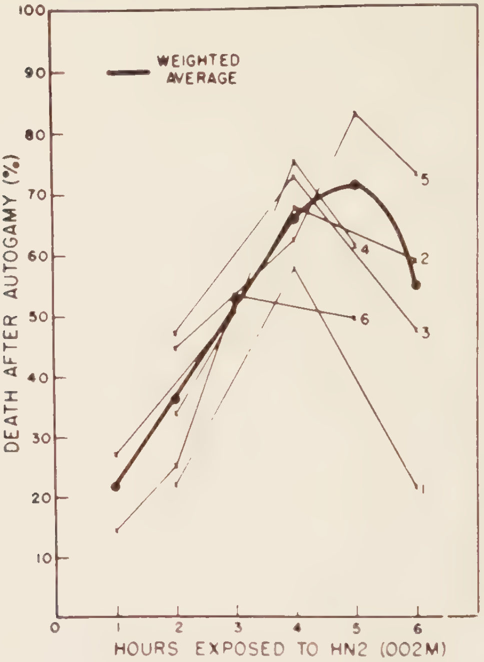

FIGURE 8. Genetic response induced by HN2 in *P. aurelia*.[33]

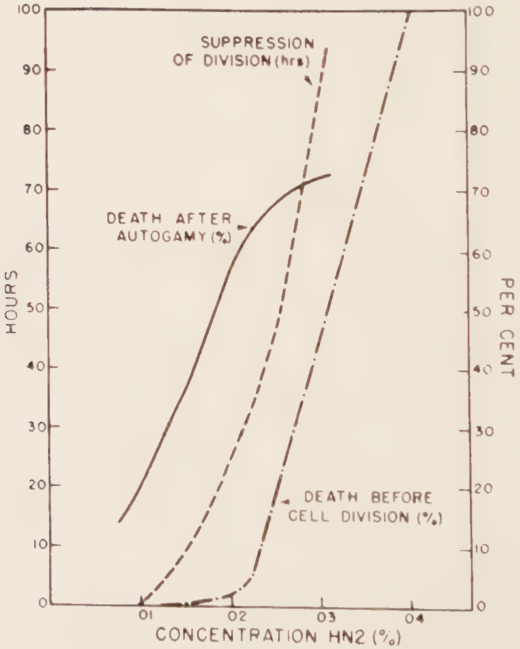

FIGURE 9. Responses in *P. aurelia* following treatment by HN2.[33]

nitrogen mustard were as follows: the concentration of nitrogen mustard necessary to bring about inhibition of fission was almost high enough to kill cells before division. Second, the return to normal rates and characteristics of fission was not immediate, but took place gradually over a number of divisions. Third, a micronuclear effect was observed in which the response came to a maximum ("peak effect") and fell off with increased dosage to the cell.

Our experiments with X rays gave the following general results: as with HN2 treatment, fission was inhibited in the region in which cell death occurred; at ordinary temperatures the LD_{50} was approximately 200,000 r. At these dosages we saw profound effects on the fission mechanisms. In general, the pattern was like that observed with nitrogen mustards. FIGURE 10 shows that the relative fission rate was low at first and then gradually increased.[31]

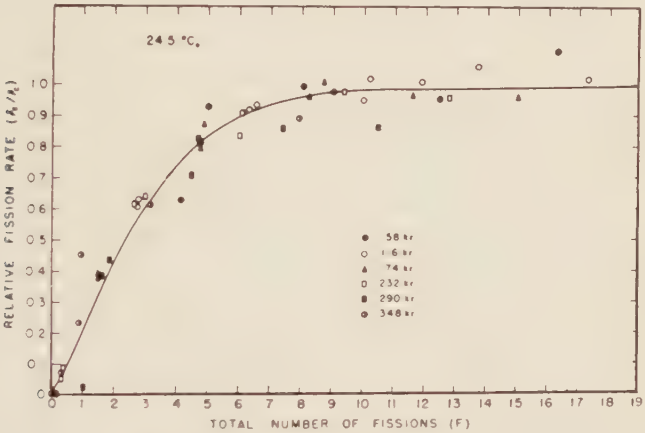

FIGURE 10. Changes in rate of cell division in *P. aurelia* following X ray.[31]

For both agents the extent of damage measured by the reduction of fission rate was a function of total dose given. Analysis of the X ray data shows, however, that the rate of recovery was independent of dose, although, of course, the time and the number of fissions necessary to attain complete recovery were directly dependent upon the degree of damage suffered.

The postautogamy response for X rays was of the same general nature as that seen after treatment with nitrogen mustard, but the dosage relationships were quite different. Postautogamous death (FIGURE 11) was induced by dosages of X rays that were small compared to those necessary to bring about cell death. Also, there was a suggestion of a peaked response such as that seen after treatment with nitrogen mustard.

The various responses to the two agents have been compared[28] as follows: the micronuclear response (FIGURE 12) became saturated quickly in comparison to inhibition of fission; that is, the micronuclear effect was induced very rapidly when compared to the amount of suppression of fission or cell

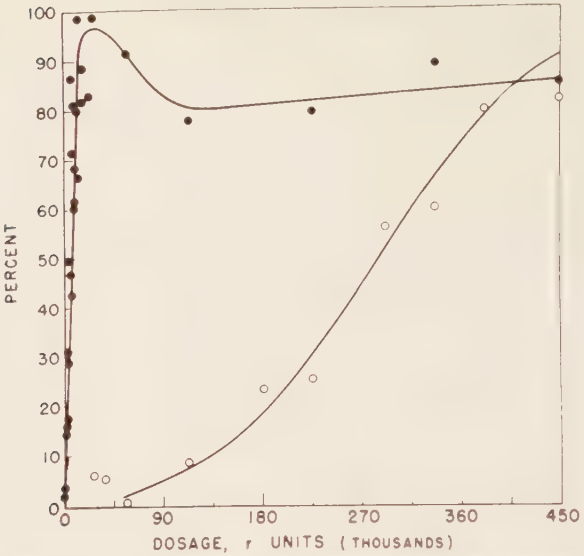

FIGURE 11. Postautogamous death (closed circles) and cell death (open circles) after X rays in *P. aurelia*.[34]

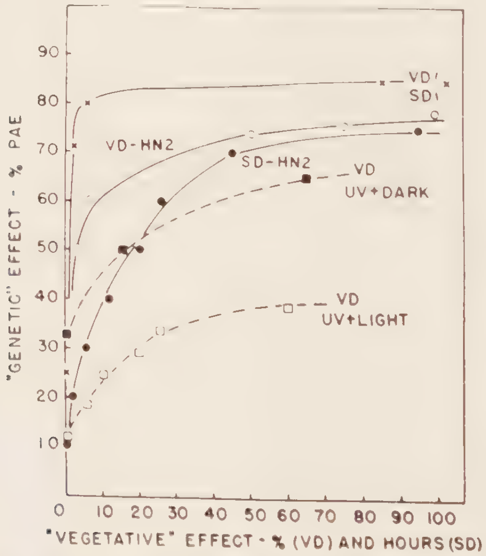

FIGURE 12. Comparison of efficiency of X rays (X) and HN2 in the induction of vegetative death (VD), suppression of fission (SD) relative to postautogamous death ("genetic effect") in *P. aurelia*.[28] UV refers to ultraviolet treatments (calculated from Kimball *et al.*[29]).

death observed at corresponding dosage levels. This is similar to the observations on amoebae, indicating that suppression of fission was due to cytoplasmic damage. Further, the effects brought about by HN2 were quantitatively different from those induced by the X rays. The so-called vegetative effects of cell death or suppression of fission occur earlier in relation to the genetic effects. The ultraviolet effects noted were calculated from the data of Kimball *et al.*[29] and showed that for ultraviolet radiations the vegetative effects were quite prominent for unit genetic effect induced by the dosage. Furthermore, we see that the nuclear effect is much more easily photoreversible than is the vegetative effect.

While one may be tempted to believe that a difference in mechanism of action is indicated by the differences in quantitative relationships among these various effects for X rays on the one hand and mustards on the other, we do not feel that this is necessarily required. Even if we postulate (Powers and Ehret[28]) that X rays and the alkylating agents affect the same essential cellular components in a similar manner, we can still expect that there will be significant differences in relative effects. X rays cause primary ionizations that should be distributed equally throughout the cell; on the other hand, the alkylating agents must diffuse into the cell from the outside, and so there would always be a higher concentration in the cytoplasm than in the nucleus. Effects primarily controlled by cytoplasmic events would be proportionately more sensitive to nitrogen mustards; effects primarily controlled by nuclear events would need higher external dosages of nitrogen mustards to achieve the same response (the precise role of the macronucleus in these effects remains to be elucidated).

We conclude, therefore, that: (1) suppression of fission is brought about by damage to cytoplasmic entities; (2) the amount of damage is in proportion to the concentration of active agent; (3) concentration of mustards in the cytoplasm is higher than in the nucleus; (4) the concentration of ions produced by X irradiation in the cytoplasm is the same as in the nucleus; (5) suppression of fission is brought about only by high concentration of active agent in the cytoplasm; and (6) therefore, the apparent difference between the two agents in inducing suppression of fission does not indicate differences in sites of action but, rather, differences in relative distributions of the two agents.

Summary

It is difficult to compare meaningfully the amount of electromagnetic radiation applied to a cell with the amount of an added alkylating agent, since these are two entirely dissimilar external influences. We cannot always know the amount of active reagent introduced into a biological system by measuring the extent to which that system is changed. If, however, two different treatments produce equivalent amounts of biological effect, some approximate calculations of the ratios of active material in the two instances can be made.

Knowing the amount of added alkylating agent, we can calculate the number of molecules of reagent per unit volume in the culture medium. It is not as easy to do this for ionizing radiation, since we still have much to learn about the nature and numbers of the different possible reaction products when radiation interacts with simple systems such as water. It is now accepted that both ions and excited molecules play an active part in radiochemical reactions. The primary event is the ejection of high-energy electrons from atoms by the photons; both high-energy and lower-energy electrons can cause excitations of various kinds in different molecules. In turn, these activated molecules, as well as the original ionized one, may undergo subsequent chemical change decomposition, rearrangement, or recombination to give new molecules. It is only relatively recently that precise measurements have been made on simple inorganic systems, such as oxidation of ferrous to ferric ion. For more complex organic materials, which decompose to a wide variety of products, quantitation is more difficult.

A reasonable estimate of the number of primary ionizations in water is about 1.8×10^{12} ionizations per gram-roentgen. One roentgen, therefore, produces a solution that is 3×10^{-9} molar in ion pairs. We may use this to compare the relative efficiency of other treatments, such as the addition of solutions of nitrogen mustards of known molarity with no implications as to the number of molecules that each active species affects.

TABLE 1

RELATIVE EFFECTIVENESS OF IONIZING RADIATION AND HN2 IN BIOLOGICAL SYSTEMS

Organism	Effect	$\frac{\text{Molarity ion pairs}}{\text{Molarity HN2}} = M_{ip}/M_n = R$
Amoeba[24–27]	LD_{50} nuclei	36
Amoeba[24–27]	LD_{50} cytoplasm	8.70
Chilomonas[3–6]	Fission delay	0.60
Pelomyxa[8–10]	Fission delay	2.10
Paramecium[29]	Fission delay	0.14
Paramecium[1, 28, 30]	Preautogamous (vegetative) death to 50 per cent level	0.25 (HN2-amine)
		0.017 (HN2-imine)
	Postautogamous ("genetic") death to 50 per cent level	0.0034 (HN2-amine)
		0.001 (HN2-imine)
	Seventy-two-hour fission delay	0.09 (HN2-amine)
		0.014 (HN2-imine)

In TABLE 1 we compare for various organisms definite biological effects obtained both by treatment with the nitrogen mustard HN2 and by X irradiation. We calculated R, the ratio of the molarity in ion pairs to the molarity of the nitrogen mustard solution to which the organisms were exposed. This, of course, gives no information about the concentrations of the active molecules of HN2 inside the cells. The larger R is, the more sensitive the particular biological system is to nitrogen mustard; the smaller R is, the more sensitive, proportionately, to X rays.

Obviously there is a wide spread of values, even for the same effect. For instance, the nuclear effects in *Paramecium* and in *Amoeba* yield values differing by 36,000-fold. Apparently the difficulties in quantitation and, perhaps, differences among cells, do not allow fruitful inquiry along these lines at the present time. The effects brought about by the substances are concentration-dependent, however, so that comparisons of this type should be possible. Our inability to make the comparisons underlines our ignorance of what basic mechanisms in the cell are affected, and how their sensitivities may vary from situation to situation. It can be pointed out that the values observed in *Paramecium* are those expected if fission suppression is due to cytoplasmic damage, if postautogamous death is due to micronuclear damage, and if the nitrogen mustard exists in higher concentration in the cytoplasm than in the micronucleus.

TABLE 2
SUMMARY OF EFFECTS ON CELL DIVISION

Effect		HN2	X rays	
			Single dose	Constant dose
Inhibition	Immediate	*P. carolinensis*—unfed[8] *P. aurelia*—unfed[1, 28] *A. proteus*—cytoplasm[25, 26] Yeast[11]	*Chilomonas*—exp.[5, 6] *P. carolinensis*—unfed[8] *P. aurelia*—unfed[28, 30] *A. proteus*—nucleus and cytoplasm[27]	Yeast[13, 18]
	Delayed	*Chilomonas*—exp.[3] *P. carolinensis*—fed[8] *P. aurelia*—fed[8] *A. proteus*—nuclei[25, 26]	*P. illinoisensis*[10] *P. aurelia*—fed[29]	*E. coli*—exp.[7]
Recovery rate above control rate		*Chilomonas*[3] *P. carolinensis*[8]		
Control fission rate in presence of agent		*Chilomonas*[3]		*E. coli*[7]
Synthesis during block		*Chilomonas*—N.A. and protein[4] *E. coli*—dry weight[11] *E. coli*—RNA, DNA*[12] Yeast—protein and dry weight†[13, 18]	*Chilomonas*—starch[5, 6]	*E. coli*—dry weight and N.A.[7]
Cell death in inhibiting dosage range		*Chilomonas*[3] *P. aurelia*[1, 28, 29] *A. proteus*—nucleus in cytoplasm[25, 26] Yeast †[3, 18]	*Chilomonas*[5, 6] *P. aurelia*[1, 28, 29] *A. proteus*—cytoplasm[27] Yeast[3, 18]	
"Peaked" response (nuclear change)		*P. aurelia*[23]	*P. aurelia*[28]	

Symbol: exp. = exponential growth.
* Diazouracil used.
† TEM used.

However, order and system exist in the manner in which the various kinds of cells tested respond to the two different agents. If judgments and comparisons are made on a qualitative basis, the orderly relationships shown in TABLE 2 emerge. In this table a number of organisms show unexpected uniformity of response to the agents.

Inhibition pattern. Immediate or delayed inhibition may be seen in all the experimental cells after either treatment. After HN2 treatment, immediate delays are observed in starved animals, while a fission or two may be seen prior to inhibition when the cells are not starved. This is true also of the response of *P. aurelia* after X rays. It seems that if the biochemical supply lines are filled, division will proceed even after the sensitive spots have been injured.

Recovery rate. Rates of cell multiplication exceeding control rates have been observed in only two species (information from studies on tissue cultures based on the uncorrected mitotic index is not considered reliable evidence for "overcompensation"). However, few studies permit recognition of this if it occurs. In the case of *Chilomonas*, the cells are very large, and the increased rate may reflect the presence of excessive amounts of the metabolites governing rate at the time division begins.

Fission in presence of agent. This is a significant observation from two different studies on different organisms, with different agents. The results are almost identical. For obvious reasons, this effect should be sought in other studies that allow its recognition.

Synthesis during block. The synthesis of nucleic acid, protein, and starch is commonly observed in cells that cannot divide following HN2 and X-ray treatment. This fact should be considered and explained by those who ascribe particular biological value to reactions *in vitro* between active agents and biochemical substances. If, for instance, the action of alkylating agents in cells is a consequence of reaction between them and nucleic acids, why should protein synthesis, commonly believed to be under RNA control, continue in cytoplasm with high HN2 concentration?

Cell death and inhibition of fission. In most of the organisms tested, and for both agents, cell death occurred in the dose region at which fission inhibition was observed. This suggests that for both agents the cause of death and the cause of fission block were closely related. The one instance (Powers and Ehret[28]) in which a nuclear effect and other effects were measured simultaneously indicates that, for unit nuclear effect, cell death and fission suppression is high after mustard treatment and low after X-ray treatment. This result is expected if the basis for cell death and fission suppression are cytoplasmic, for mustard treatment must result in high cytoplasmic concentrations for unit nuclear effect.

"Peaked" nuclear response. After both agents, a curious "peaked" effect was observed in *P. aurelia*. Its significance will be discussed elsewhere.

Acknowledgement

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References

1. Powers, E. L., C. A. Raper & J. H. Pomeroy. 1954. Biological effects of certain hydrolysis products of a nitrogen mustard in *Paramecium aurelia*. Arch. Biochem. Biophys. **56**: 297–306.

2. Visconti di Modrone, N. & M. Simonetta. 1949. Azione mutagena delle sistanze chimiche. Nota III. Cinetica della azione letale della metil-$\beta\beta'$ didcloroetil ammina su *Escherichia coli*. Gazz. chim. ital. **79**: 822–831.
3. Hutchens, J. O. & B. Podolsky. 1954. The effect of nitrogen mustards on cell division in *Chilomonas paramecium* and *Saccharomyces cerevisiae*. J. Cellular Comp. Physiol. **43**: 205–228.
4. Podolsky, B. & J. O. Hutchens. 1954. Some effects of nitrogen mustards on metabolism of *Chilomonas paramecium* and *Saccharomyces cerevisiae*. J. Cellular Comp. Physiol. **43**: 229–246.
5. Ducoff, H. W. 1953. Factors influencing the duration of postirradiation cell division block in *Chilomonas paramecium*. Dissertation. Univ. Chicago. Chicago, Ill.
6. Ducoff, H. S. 1957. Factors affecting radiation-induced division delay in *Chilomonas paramecium*. Physiol. Zool. **30**: 268–280.
7. Rubin, B. A. 1954. Growth and mutation of bacteria during continuous irradiation. J. Bacteriol. **67**: 361–368.
8. Daniels, E. W. 1952. Some effects on cell division in *Pelomyxa carolinensis* following X-irradiation with bis(2-chloroethyl) methylamine and experimental plasmogamy (fusion). J. Exptl. Zool. **120**: 509–523.
9. Daniels, E. W. 1954. Cell division in the giant amoeba, *Pelomyxa carolinensis*. II. Analysis of therapeutic effects after fusion with non-irradiated cell portions. J. Exptl. Zool. **127**: 427–461.
10. Daniels, E. W. 1955. X-irradiation of the giant amoeba, *Pelomyxa illinoisensis*. J. Exptl. Zool. **130**: 183–197.
11. Loveless, L. E., E. Spoerl & T. H. Weisman. 1954. A survey of effects of chemicals on division and growth of yeast and *Escherichia coli*. J. Bacteriol. **68**: 637–644.
12. Katchman, B. J., E. Spoerl & H. E. Smith. 1955. Effects of cell division inhibition on phosphorus metabolism of *Escherichia coli*. Science. **121**: 97–98.
13. Spoerl, E., L. E. Loveless, T. H. Weisman & R. J. Balske. 1954. Studies on cell division. II. X-radiation as a division inhibiting agent. J. Bacteriol. **67**: 394–401.
14. Kinsey, V. E. & W. M. Grant. 1947. Action of mustard gas and other poisons on yeast cells. I. Effect of mustard gas on the rate of cell division. J. Cellular Comp. Physiol. **29**: 51–64.
15. Kinsey, V. E. & W. M. Grant. 1947. Action of mustard gas and other poisons on yeast cells. II. Effect of mustard gas on the mortality, morphology, carbohydrate metabolism, and permeability. J. Cellular Comp. Physiol. **29**: 65–74.
16. Kinsey, V. E. & W. M. Grant. 1947. Action of mustard gas and other poisons on yeast cells. III. Distribution of fixed mustard gas in yeast. J. Cellular Comp. Physiol. **29**: 75–84.
17. Brace, K. C. 1950. Effects of X rays on size of yeast cells. Proc. Soc. Exptl. Biol. Med. **74**: 751–755.
18. Spoerl, E. & R. Carleton. 1954. Studies on cell division. Nitrogen compound changes in yeast accompanying an inhibition of cell division. J. Biol. Chem. **210**: 521–529.
19. Burns, V. W. 1956. X-ray induced division delay in individual yeast cells. Radiation Research. **4**: 394–412.
20. Mortimer, R. K. 1955. Evidence for two types of X-ray induced lethal damage in *Saccharomyces cerevisiae*. Radiation Research. **2**: 361–368.
21. Wood, T. H. 1953. Reproducibility of X-ray survival curves for yeast cells. Proc. Soc. Exptl. Biol. Med. **84**: 446–452.
22. Zirkle, R. E. & C. A. Tobias. 1953. Effects of ploidy and linear energy transfer on radiobiological survival curves. Arch. Biochem. Biophys. **47**: 282–306.
23. Harriss, E. B., L. F. Lamerton, M. J. Ord & J. F. Danielli. 1952. Site of action of mutagenic reagents; X-rays. Nature. **170**: 921.
24. Ord, M. J. & J. F. Danielli. 1952. Site of action of mutagenic reagents; nitrogen mustards. Nature. **170**: 921.
25. Ord, M. J. 1956. The site of damage in amoebae exposed to low concentrations of methyl di-(β-chloroethyl)-amine (a 'nitrogen mustard'). Quart. J. Microscop. Sci. **97**: 39–45.

26. ORD, M. J. & J. F. DANIELLI. 1956. The site of damage in amoebae exposed to lethal concentrations of methyl di-(β-chloroethyl)-amine (a 'nitrogen mustard'). Quart. J. Microscop. Sci. **97**: 17–28.

27. ORD, M. J. & J. F. DANIELLI. 1956. The site of damage in amoebae exposed to X-rays. Quart. J. Microscop. Sci. **97**: 29–37.

28. POWERS, E. L. & C. F. EHRET. 1956. Radiation effects on cells: genetics or physiology? *In* Biological Effects of Radiation. Proc. Intern. Conf. on Peaceful Uses of Atomic Energy. **11**: 266–272. United Nations. New York, N. Y.

29. KIMBALL, R. F., R. P. GECKLER & N. GAITHER. 1952. Division delay by radiation and nitrogen mustard in *Paramecium*. J. Cellular Comp. Physiol. **40**: 427–459.

30. POWERS, E. L. 1955. Radiation effects in *Paramecium*. Ann. N. Y. Acad. Sci. **59**(4): 619–636.

31. SACHER, G. A., E. L. POWERS, D. SHEFNER & J. M. GURIAN. 1954. Effects of X rays on division of *Paramecium aurelia*. Radiation Research. **1**: 228.

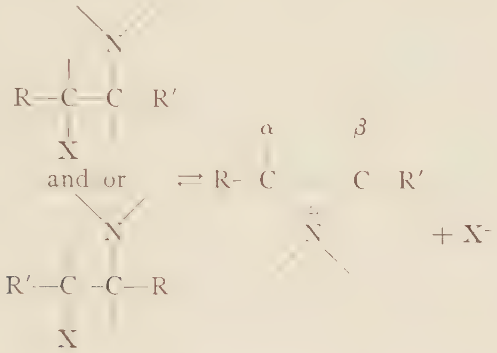
32. GECKLER, R. P. 1950. Genetic changes induced by exposure to nitrogen mustard and their inheritance in *Paramecium aurelia*, variety 4. Genetics. **35**: 253–277.

33. POWERS, E. L. & C. A. RAPER. Unpublished.

34. POWERS, E. L. & D. SHEFNER. Unpublished.

DISCUSSION: PART I

Norman H. Cromwell (*Department of Chemistry, University of Nebraska, Lincoln, Nebr.*): In connection with Ross's paper, I should suggest that it seems more logical always to write the structure for the ions resulting from most β-chloroethylamines in some sort of cyclic arrangement rather than as solvated carbonium ions.



Stereochemical and kinetically controlled ring cleavage reaction experiments with ethylenimines and ring closures and rearrangements with the resulting β-chloroethylamines give clear indication that each ring cleavage and each ring closure in the series studied was accompanied by a Walden inversion, whether the conditions were such as to favor an S_N1- or an S_N2-type reaction. This indicates a steric control by the amino group, best represented by a three-ring arrangement showing some bonding to both the α and β carbons.[1-3]

In the case of ring cleavages of ethylene oxides with amines, a kinetic study has established second-order kinetics for one such system.[4]

Concerning the ingenious and valuable suggestion in the paper by Stacey *et al.*, that alkylation with the primary phosphate groups rather than with other groupings in DNA is the important reaction that destroys its ability to function, one realizes that such esterifications may be reversed through saponification. This possibility provides a chemical flexibility not possible with concepts involving nonreversible alkylations on nitrogen and sulfur. Such opportunities for reversal of alkylation might conceivably be associated with the resistance problem encountered in the application of alkylating agents to the control of tumor growth.

References

1. Dickey, F. H., W. Fickett & H. J. Lucas. 1952. J. Am. Chem. Soc. **74**: 944.
2. Cromwell, N. H., G. V. Hudson, R. A. Wankel & P. J. Vanderhorst. 1953. J. Am. Chem. Soc. **75**: 5384.
3. Cromwell, N. H. & A. Hassner. 1955. J. Am. Chem. Soc. **77**: 1568.
4. Barker, N G. & N. H. Cromwell. 1951. J. Am. Chem. Soc. **73**: 1051.

J. J. Roberts (*Chester Beatty Research Institute, Royal Marsden Hospital, London, England*): I propose to report on some work being carried out jointly with my colleague, G. P. Warwick,[1] which has provided direct evidence of *in vivo* alkylation in the case of these biologically active compounds. With this concept, however, it must be assumed that the functional group primarily involved in the production of the biological effects may not necessarily be the phosphate group. Ross's view is based, as he has stated, on an elimination of the other possible reaction sites--possible, that is, on purely chemical grounds. On the basis of our results, however, we do not feel that all groups other than phosphate may be so readily dismissed.

In order to obtain some precise information about the mode of action of these alkylating agents, we have studied the metabolic products and the distribution in the rat of a radioactive tumor-inhibiting compound. The compound chosen was one of a series of monofunctional methanesulfonyloxy esters that have recently become available and have been found to be active in inhibiting the growth of the transplanted Walker rat carcinoma.[2] Of the many compounds tested in this series, only those shown in TABLE 1 had any activity; that of CB 1506, chloroethyl methanesulfonate, was markedly higher than that of the others shown. I shall present later an explanation for the somewhat unexpected and anomalous activity of this compound. The compound actually used was ethyl methanesulfonate, CB 1528, which is closely related to the now familiar series of dimesyloxy esters, of which Myleran ($n = 4$) is the most active. CB 1528 is, in fact, a half Myleran.

Table 1

Tumor-Growth-Inhibiting Alkyl Methanesulfonates

$CH_3SO_2{\cdot}O(CH_2)_nX$

$n = 2$	$x = Cl$	CB 1506
$n = 2$	$x = F$	CB 1522
$n = 2$	$x = H$	CB 1528
$n = 1$	$x = H$	CB 1540

$CH_3{\cdot}SO_2{\cdot}O(CH_2)_n{\cdot}O{\cdot}SO_2{\cdot}CH_3$

$n = 4 =$ Myleran.

It is worth mentioning here that monofunctional alkylating agents are generally effective only at concentrations 50 to 100 times those of the difunctional or polyfunctional compounds. It was felt, however, that the *in vivo* reactions of these compounds, in which we were particularly interested, would be comparable to those of the difunctional ones and would permit a more facile determination of the reaction products.

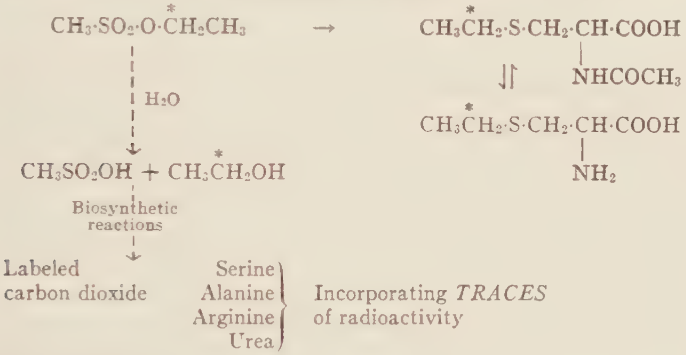
C^{14}-labeled ethyl methanesulfonate was injected intraperitoneally into normal and tumor-bearing male Wistar rats, and the nature of the radioactive urinary constituents was investigated. In collaboration with W. Davis, we also investigated the distribution of radioactivity in the rat at the end of 24 hr. Twenty-five to 35 per cent of the activity was exhaled as labeled carbon dioxide, 1 to 2 per cent appeared in the feces, and only 5 to 8 per cent remained bound in the rats. This remainder was generally distributed throughout all the organs. Twelve to 15 per cent of the radioactivity appeared in the urine during the first 24 hr., 4 per cent during the next

48 hr., and negligible quantities from then on. The remaining activity is due to volatile reaction products other than carbon dioxide or to unchanged drug that would not be detected by the counting methods so far employed.

I shall restrict my remaining comments to the nature of the radioactive constituents that appeared in the first 24-hr. urine sample. These were determined using the techniques of paper chromatography, subsequent autoradiography of paper chromatograms, and by means of ion exchange chromatography. The results are shown in TABLE 2.

Table 2

Rat Urinary Constituents Containing C^{14}, 24 Hr. after Injection (I.P.) of Ethyl Methanesulfonate


$CH_3{\cdot}SO_2{\cdot}O{\cdot}\overset{*}{C}H_2CH_3$
$CH_3\overset{*}{C}H_2{\cdot}S{\cdot}CH_2{\cdot}CH(NHCOCH_3){\cdot}COOH$
$CH_3\overset{*}{C}H_2{\cdot}S{\cdot}CH_2{\cdot}CH(NH_2){\cdot}COOH$
H_2O
$CH_3SO_2OH + CH_3\overset{*}{C}H_2OH$
Biosynthetic reactions
Labeled carbon dioxide
Serine
Alanine
Arginine
Urea
Incorporating *TRACES* of radioactivity

* Labeled carbon atom.

Traces of radioactivity were associated with the amino acids, serine, alanine, and arginine, and with urea. It seems likely that radiocarbon was incorporated into these compounds following hydrolysis of the drug into radioethanol, which was then involved in biosynthetic reactions. The rapid excretion of large quantities of labeled carbon dioxide also probably resulted from this process. This view was confirmed by autoradiography of paper chromatograms of urine from rats injected with radioethanol equivalent in amount to that which theoretically would be liberated on hydrolysis of all the injected drug. Radioactivity was again found only in amino acids and urea.

However, it was shown that the bulk of the radioactivity was present in two newly present sulfur-containing acids, one of which was the mercapturic acid, N-acetyl-S-ethyl cysteine (TABLE 2), and the other was almost certainly a conjugate or derivative of it from which we have shown it may be formed. Paper chromatograms of acid-hydrolyzed urine indicated activity corresponding almost entirely to S-ethyl cysteine. Two other traces of activity that could sometimes be detected, and that gave brown spots with ninhydrin in the presence of dicyclohexylamine, again appeared to be due to metabolic products of S-ethyl cysteine, since chromatograms of urine similarly obtained from rats injected with S-ethyl cysteine also contained these compounds.

We found no labeled carboxylic or phosphoric acid esters or alkylated amines in the urine; these might have been present if the drug reacted with acids or amines *in vivo*. However, the possibility that they were formed initially was not precluded, since they could be further metabolized prior to excretion. Nevertheless, to summarize, the fact remains that a large proportion of the drug had been involved in alkylation of the thiol group *in vivo*. This appeared to be the first *in vivo* evidence of plain alkylation in the case of these biologically active alkylating agents.

What, then, can be deduced with regard to the mode of action of the drug from such a result? A number of questions at once suggest themselves; some of these we are in a position to answer. First, is the presence of ethyl cysteine per se, or one of its metabolites, responsible for the observed biological effects of CB 1528? In this connection it is interesting to recall that ethionine, that is, S-ethyl homocysteine, inhibits the growth of the Jensen sarcoma,[3] probably because of its ability to interfere with transmethylation reactions and protein synthesis. As far as the inhibition of the Walker tumor is concerned, however, it has clearly been shown by Haddow[4] that S-ethyl cysteine is quite inactive.

Second, is the process of forming the S-ethyl cysteine *in vivo* responsible for the observed biological effects? In turn, this raises the question as to the source of the cysteine in mercapturic acid formation. This question has fascinated biochemists for twenty years and the answer is still not definitely known, although there is ample evidence to suggest that protein-bound cysteine or glutathione cysteine could contribute to the formation of mercapturic acid.[5] Thus, mercapturic acid excretion has been demonstrated in rabbits fed bromobenzene after a thirty-seven-day starvation period. Again, rats fed labeled cysteine and bromobenzene excreted a far smaller proportion of the labeled mercapturic acid than would be anticipated had it been formed largely from free cysteine. It is also pertinent to note that H. G. Crabtree, during his carcinogenesis experiments, obtained evidence for the excretion of mercapturic acid in the urine after application of bromobenzene to the skin, there being a corresponding decrease in the sulfhydryl concentration at the point of application.

As far as the *in vitro* work is concerned, it is more than adequate to suggest a facile reaction between the thiol groups of proteins and alkylating agents.[6, 7] Alexander[8] has shown recently that the thiol groups in a reduced protein have a very favorable competition factor by comparison with the carboxyl and amino groups in reactions with ethyl methanesulfonate, the compound used in our investigations.

I digress for a moment here to offer an explanation for the apparent anomaly that exists with regard to the reactions between alkylating agents and the protein SH group which, with its apparently high *p*Ka value, should exist largely in the unreactive un-ionized form. It is clear that the character of every SH group within a protein structure is unique, its properties being dependent on its particular environment within that structure, which will affect not only its *p*Ka value, but also its steric availability. Herein lies an explanation for the known diverse pharmacological effects of a whole range of SH reactants, including, indeed, the different types of biological alkylating agents to which Bacq[7] has given the name thiolprives; all of these could be exhibiting a selectivity for particular SH groups. Clearly, the *in vivo* picture is even more complicated, with contiguous protein fibers also contributing to the SH environment.

What, then, might be the consequences of reaction with body sulfhydryl groups, whether they are the free or soluble thiol groups, as in cysteine, glutathione, or such cofactors as coenzyme A, or the fixed SH groups, as in protein (any such reactions being, in view of the *in vitro* and now the *in vivo* evidence, both possible and, indeed, eminently likely)?

It has been shown that compounds excreted as mercapturic acids, such as bromobenzene and certain hydrocarbons, all tend to inhibit the growth of an animal when administered in large quantities. The mechanism is thought to be one involving an interference with sulfur metabolism in general by depletion of body cysteine, since addition of sulfur-containing amino acids will reverse the process. However, it seems highly unlikely that the type of activity manifested by the difunctional alkylating agents, whereby their effects are apparent at much smaller concentrations, is due to a similar mechanism.

A second possible consequence of reaction with protein sulfhydryl groups is an inactivation of essential sulfur-containing enzymes. This mechanism of action of the alkylating agents has been rejected by some on the grounds that not all SH enzymes are inhibited *in vitro*. I do not wish to discuss this point here, apart from mentioning, again for the reasons stated earlier, that there is no truly specific SH reagent or, indeed, specific SH enzyme inhibitor, so that one would not expect all SH enzymes to be inhibited by any one compound. Furthermore, there is no absolute correlation between *in vitro* and *in vivo* enzyme studies.

However, quite apart from an interference with energy-yielding biosynthetic processes in which the thiol group has been shown clearly to be important, reaction with protein SH could interfere with its function in what Mazia[9] describes as the "role of the thiol group in the structural machinery which carries out the process of cell division." Here I wish to recall the classic work of L. Rapkine, repeated and developed in recent years by Mazia, on the relationship between SH concentrations and cell division. It has been found that the concentration of free SH groups in the cell varies throughout the mitotic cycle of the freshly fertilized sea urchin egg, such variation being reflected by opposite changes in the concentration of the protein or fixed SH groups. The concentration of protein SH groups is at maximum during the resting stage, when it is thought that denaturation of the protein, leading to exposure of hitherto concealed thiol groups occurs.

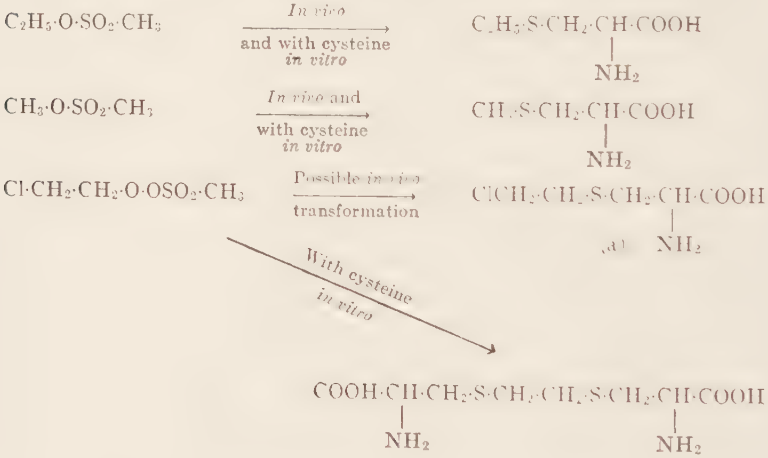
Revell[10] has shown that only cells treated at the earlier part of the resting stage of the cell cycle with certain alkylating agents in the next mitosis showed chromosome aberrations. We feel it would be not too speculative to suggest a correlation between these two findings that could explain not only the apparent specificity that such compounds exhibit for dividing cells, but also this particular sensitivity within the cell cycle. A reaction with the SH group of nucleoprotein or, more especially, a crosslinking reaction between two such molecules could well explain the observed cytological and mutagenic effects. After all, it still seems necessary to find a mechanism of action that explains the enhanced activity of the difunctional compounds.

I conclude by mentioning one interesting corollary that arises from the demonstrated reaction between methanesulfonyloxy esters and a sulfhydryl group *in vivo* and that could provide an explanation for the remarkable biological effects of the closely related chloroethyl methanesulfonate, CB 1506, as shown in TABLE 1. This compound was quite unlike other methanesulfonyloxy esters in its mutagenic effects on *Drosophila melanogaster*.

Reaction with cysteine in a manner analogous with that found in the case of both ethyl methanesulfonate and methyl methanesulfonate (which subsequently we showed to react similarly) would, in the case of the chloroethyl compound, give, in the first instance, S-chloroethyl cysteine, a monofunctional sulfur mustard, with an effective amino acid-carrying structure (TABLE 3a). On the other hand, if reaction occurred with a bound SH group, one would have created, in effect, a difunctional compound *in situ* favorably placed for further reaction.

TABLE 3

In Vitro AND *In Vivo* TRANSFORMATIONS OF ALKYL METHANESULFONATES



$$C_2H_5{\cdot}O{\cdot}SO_2{\cdot}CH_3 \xrightarrow[\text{and with cysteine } in\ vitro]{In\ vivo} C_2H_5{\cdot}S{\cdot}CH_2{\cdot}\underset{NH_2}{\underset{|}{CH}}{\cdot}COOH$$

$$CH_3{\cdot}O{\cdot}SO_2{\cdot}CH_3 \xrightarrow[\text{with cysteine } in\ vitro]{In\ vivo \text{ and}} CH_3{\cdot}S{\cdot}CH_2{\cdot}\underset{NH_2}{\underset{|}{CH}}{\cdot}COOH$$

$$Cl{\cdot}CH_2{\cdot}CH_2{\cdot}O{\cdot}OSO_2{\cdot}CH_3 \xrightarrow[\text{transformation}]{\text{Possible } in\ vivo} ClCH_2{\cdot}CH_2{\cdot}S{\cdot}CH_2{\cdot}\underset{NH_2}{\underset{|}{CH}}{\cdot}COOH \quad (a)$$

$$\xrightarrow[in\ vitro]{\text{With cysteine}} COOH{\cdot}\underset{NH_2}{\underset{|}{CH}}{\cdot}CH_2{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}S{\cdot}CH_2{\cdot}\underset{NH_2}{\underset{|}{CH}}{\cdot}COOH$$

If it appears that I have stressed the importance of the thiol group to the exclusion of all others, I hope that, rather than show my own prejudices, it may demonstrate the multiplicity of interpretation that is still possible when considering the properties and possible mode of action of the alkylating agents.

References

1. ROBERTS, J. J. & G. P. WARWICK. 1957. Studies on the mode of action of alkylating agents—the formation of S-ethyl cysteine from ethyl methanesulphonate. Nature. **179**: 1181.

2. Haddow, A. & W. C. J. Ross. 1956. Tumour growth inhibitory alkyl sulphonates. Nature. **177**: 995.
3. Levy, H. M., G. Montanez, E. A. Murphy & M. S. Dunn. 1953. Effect of ethionine on tumor growth and liver amino acids in rats. Cancer Research. **13**: 507.
4. Haddow, A. Personal communication.
5. Gutman, H. R. & J. L. Wood. 1950. The effect of bromobenzene and 3,4-benzpyrene on the metabolism of radioactive L-cystine. Cancer Research. **10**: 8.
6. Banks, T. E., J. C. Boursnell, G. E. Francis, F. L. Hopwood & A. Wormall. 1946. Studies on mustard gas (*ββ′* dichlorodiethyl sulphide) and some related compounds. IV. Their action on proteins studied with the aid of radioactive sulphur. Biochem. J. **40**: 745.
7. Bacq, Z. M. 1946. Substances thiolprives. Experientia. **2**: 349.
8. Alexander, P. Personal communication.
9. Mazia, D. 1954. SH and growth. *In* Glutathione—A Symposium. Academic Press. New York, N. Y.
10. Revell, S. H. 1953. Chromosome breakage by X-rays and radiomimetic substances in *Vicia*. Heredity. **6** (Suppl.): 107.

G. M. Timmis (*Chester Beatty Research Institute, Royal Marsden Hospital, London, England*) and R. F. Hudson (*Queen Mary College, University of London, London, England*): The clinical success of 1,4-dimethanesulfonoxybutane (Myleran, I, $n = 4$, in figure 1) in the treatment of chronic myeloid leukemia has led us to investigate possible mechanisms of action and to seek through modifications of structure substances of potential value in acute leukemia.

As a means of modifying the alkylating properties of Myleran and its congeners we added methyl groups to the terminal carbon atoms to give the series II, in the expectation that the mechanism of alkylation would be thereby changed from a largely S_N2 to an S_N1 type. This proved to be the case. One consequence of this difference would be that the values for the competition factors, which show the relative rates of reaction with a particular series of anions, would be expected to lie closer together with compounds of series II than with those of series I.[1-4] This effect, considered in relation to the anions of possible biological importance—that is, carboxylate, ionized sulfhydryl or phosphate—might, it was thought, confer some interesting difference in biological response as between series I and II. A marked difference has been found in that while very marked inhibition of the Walker tumor is produced by some members of series I[5] at doses that are tolerated by the bone marrow, no marked tumor inhibition is caused by doses of the corresponding members of series II that are probably close to the limit of toleration.[6] On a dose basis, compounds of series II are two to three times more active than those of series I[7] in depressing the circulating rat neutrophils (figure 1).

Apart from any mechanism of action involving alkylation, it was just conceivable that the methanesulfonoxy groups might be capable of attachment at some site, for example, nucleic acid or protein, by hydrogen bonding. The bis-sulfone III (figure 2), which resembles Myleran (I, $n = 4$) sterically and retains at least some of its hydrogen bonding potentiality, but has no alkylating function, was therefore made. Since it was quite inactive in depressing neutrophils,[8] alkylation must be essentially involved in the action of both I and II, and an old hypothesis[9] was critically examined: this suggested that since compounds of series I vary in activity, as shown in FIGURE 1, their activity could be explained by supposing that this depended upon the formation of a ring structure, involving the polymethylene chain, by dialkylation of, for example, an amino group. When $n = 2$ and 3 such a ring would be unstable, and when $n = 6$ to 10 it would be progressively more slowly formed. Thus, the most active compounds would readily form stable rings.

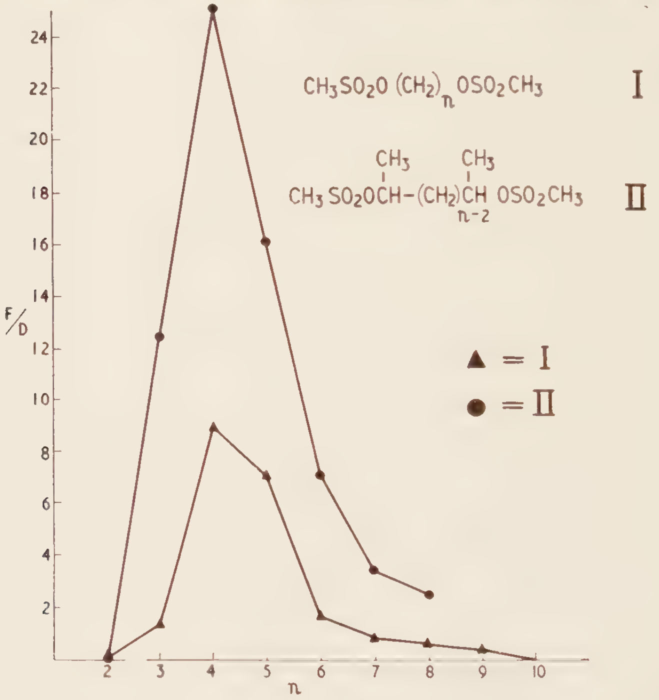


FIGURE 1. F = percentage fall in neutrophils from normal; D = dose in mg./kg.

$CH_3\ O_2S\ CH_2CH_2CH_2CH_2CH_2CH_2SO_2CH_3$

III

FIGURE 2

The conception could be applied to reaction with sulfhydryl and also with secondary phosphate groups occurring in, for example, ribonucleic acid or adenosine triphosphate, since of the cyclic esters that would be formed from I ($n = 2$, 3, or 4) and the secondary phosphate, that from I ($n = 4$) would have the most stable (seven-membered) ring.[10] Assessment of alkylating ability by measurement of solvolysis rates (FIGURE 3) showed constant values for I, $n = 4$ to 10, and the fall in biological activity over this range could have been explained solely by the cycloalkylation hypothesis but for the

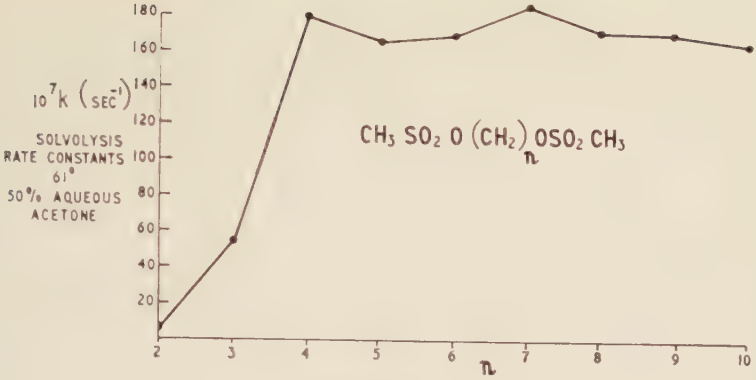


FIGURE 3

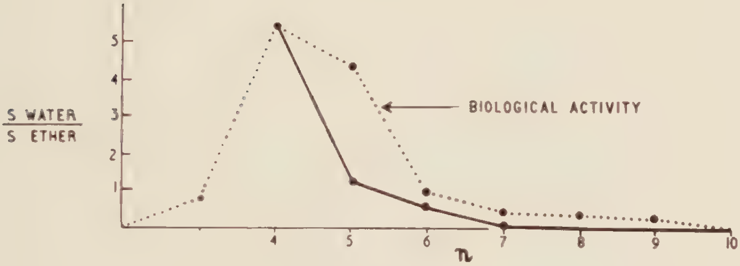


FIGURE 4

	NE	CE	TI	ND
$CH_3SO_2OCH_2.C{\equiv}C.CH_2OSO_2CH_3$	+	+	+	-

15 mg./Kg.

IV

NE = Nucleotoxic effect
CE = Cytotoxic effect
TI = Inhibition of tumor growth
ND = Neutrophil depression

FIGURE 5

fact that the ratio of solubilities in water and ether also fall in a somewhat similar way (FIGURE 4). The fall in neutrophil-depressing activity in series II (FIGURE 1) from $n = 4$ to 8 also goes along with a progressive fall in the water-ether solubility ratios. The gradual alteration in activity might therefore be related to differences in the ratio of oil and water solubilities, as suggested in the Meyer and Overton hypothesis of anesthetic action. On the other hand, an analogue of I ($n = 4$), IV (FIGURE 5), which cannot participate in cycloalkylation by reason of the triple bond in its structure, was inactive as a neutrophil-depressant, although it affected the Walker tumor[11] (FIGURE 5). The cycloalkylation hypothesis would also explain the inactivity of butyl methanesulfonate and 2-pentyl methanesulfonate, which are monofunctional analogues of I ($n = 4$) and II ($n = 4$), respectively, since the complete alkylation of the appropriate center would be much less facile than with a bifunctional compound. The same effect is, however, explicable in other ways,[12, 13] and the hypothesis remains open to doubt.

The greater solubility of II ($n = 4$) compared with Myleran and its quicker onset of action have led to trials in acute leukemia that have just been initiated.[14]

References

1. Ogston, A. G., E. R. Holiday, J. St. L. Philpot & L. A. Stocken. 1948. Trans. Faraday Soc. **44**: 45.
2. Ross, W. C. J. 1949. J. Chem. Soc. : 2590.
3. Swain, C. G. & C. B. Scott. 1953. J. Am. Chem. Soc. **75**: 141.
4. Swain, C. G., C. B. Scott & K. H. Lohmann. 1953. J. Am. Chem. Soc. **75**: 136.
5. Haddow, A. & G. M. Timmis. 1953. Lancet. **1**: 207.
6. Haddow, A. 1956. Unpublished data.
7. Elson, L. A. 1958. Ann. N. Y. Acad. Sci. **68**(3): 832.
8. Elson, L. A. 1958. Ann. N. Y. Acad. Sci. **68**(3): 826.
9. Timmis, G. M. 1951. 12th Intern. Congr. Pure & Appl. Chem. Abstr. : 334.
10. Khorama, H. G., G. M. Tener, R. S. Wright & T. G. Moffat. 1957. J. Am. Chem. Soc. **79**: 430.
11. Koller, P. 1957. Unpublished data.
12. Alexander, P. & K. A. Stacey. 1958. Ann. N. Y. Acad. Sci. **68**(3): 1225.
13. Ross, W. C. J. 1958. Ann. N. Y. Acad. Sci. **68**(3): 669.
14. Bierman, H. R., K. H. Kelly, A. G. Knudson, T. Maekawa & G. M. Timmis. 1958. Ann. N. Y. Acad. Sci. **68**(3): 1211.

Part II. Biological Effects of Alkylating Agents

MUTAGENIC EFFECTS OF ALKYLATING AGENTS

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Genetics is still an esoteric branch of knowledge. Although a connection is generally admitted between nuclear events and cancer research, review papers on the origin and therapy of cancer usually contain no more than a few casual references to genetic findings, treated as odd bits of information whose bearing on the topic is obscure. Cytology fares slightly better in this respect: the fact that chromosome breakage, cytostatic action, and cancer therapy are related is too obvious to be neglected, and pictures of broken chromosomes and disturbed mitoses can be appreciated even by the nonspecialist. Genetics and cytology, however, are but complementary approaches to the same problems, and the frequencies and consequences of chromosome breakage can often be analyzed much more reliably and thoroughly by indirect genetic methods than by direct cytological observation. Moreover, there is much to be said in favor of the hypothesis that, in at least some types of malignant disease, the primary cause is mutation, that is, an event that can be studied *only* by genetic methods.

I therefore welcome the opportunity of presenting the geneticist's point of view. It is true that only a few years ago I became aware that I had been working with alkylating agents since 1940, and I was as gratified with this discovery as was Monsieur Jourdain when he learned in his old age that he had been speaking prose all his life.

The title of my paper seems to call for more than a description of how some mutagens with alkylating ability have been found to act on genetic material. It seems to imply that the genetic effects produced by alkylating agents are in some way distinct from those exerted by other chemical mutagens, or that they are at least produced by a different mechanism. This has been inferred from chemical considerations that I am not competent to evaluate. I do not say that this assumption is not true. What I do say is that genetic findings have so far given no evidence for it. The mutagenic ability of mustard gas was discovered simultaneously with that of urethane, which is not an alkylating agent. Since then, the rapid increase in the number of known alkylating agents that are mutagens as well as carcinogens and tumor inhibitors has been paralleled by an equally rapid increase in the number of other known mutagens. Some of these, for example dimethyl sulfate and diazomethane, are alkylating compounds. Formaldehyde, a very effective mutagen, *may* act by alkylation, but probably does not do so when it produces mutations and chromosome breaks. Still others, such as phenol, peroxides, acridines, and purines, are definitely not alkylating. It is true that there is no reason for assuming that all chemical mutagens produce their effects in the same way. On the contrary, it is very unlikely that they do. However, the dividing lines between groups with different effects on the genetic material are not necessarily those postulated by the chemist. If I may venture a chemical opinion, I should like to say this: the majority of mutagens with alkylating effects are chemically very active compounds that can undergo many reactions *in vitro* and may undergo still others *in vivo*. To predict which particular kind of reaction leads to genetic changes seems to me much more hazardous in the case of such substances than in chemically more inert compounds such as the purines, for which the hypothesis that they interfere in some way with the synthesis or biological activity of nucleotides seems to be reasonable.

Genetic experiments have so far shed little light on the mechanism by which any of the alkylating agents affect the chromosomes. The only clear result is a negative one, namely, that they do not act by cross linkage, as had been suggested from chemical considerations. Monofunctional mustards, epoxide, ethylenimine, and ethylenimine picrate produce mutations in *Drosophila;* in the mold *Neurospora*, certain monofunctional mustards are even more effective genetically than the corresponding bifunctional ones. This is one of the genetic results that tend to be neglected in pharmacologically oriented reviews, where it is usually stated that monofunctional compounds, if they act at all, are only $^1/_{50}$th to $^1/_{100}$th as effective as the corresponding bifunctional ones.

Another negative result consisted of showing that lewisite and chloropicrin, both potent —SH poisons, did not produce mutations in *Drosophila*. These experiments were done at a time when the action on —SH groups was stressed in the chemical interpretation of mustard gas damage. Although negative mutation tests are of very limited validity, the results suggested that —SH poisoning plays no important role in mustard gas mutagenesis, and this agrees well with more recent chemical findings.

A different attempt to analyze the action mechanism of mustard gas was made by applying treatment in the presence and in the absence of oxygen. It was found that the production of mutations by mustard gas in *Drosophila* and of chromosomal changes by nitrogen mustard in the broad bean (*Vicia faba*) does not depend on oxygen tension during treatment. This suggests that oxidative processes are not involved; but it is as well to suspend judgment, for catalase poisoning seems to increase the frequency of mustard-gas-induced mutations in *Drosophila*, and this points to some connection with oxidation. There is obviously much scope for studying the action of chemical mutagens by varying the conditions of treatment, including pretreatment and posttreatment.

A more indirect approach consists of comparing the yield of genetic effects in different types of cell or at different stages of the cell cycle and correlating the results with existing knowledge of the biochemical processes taking place in the treated cells. The difficulty here is that so little is known about these processes. Nevertheless, such studies yield results that are of interest in their own right. In mitotic plant cells, chromosome breakage by nitrogen mustards and epoxides takes place only during interphase. This has been taken to indicate a connection with the synthesis of deoxyribonucleic acid

(DNA), but this conclusion is far from compelling. Moreover, in *Drosophila* mustard gas acts very effectively on spermatozoa stored in the seminal receptacles of the female, that is, at a stage when certainly no DNA synthesis takes place. The peak of sensitivity to mustard gas in the spermatogenesis of *Drosophila* occurs prior to the formation of mature sperm, probably during the spermatid stage, and the same appears to be true for some other alkylating agents. It is interesting that it is this stage, or a very closely neighboring one, that also shows peak sensitivity to the mutagenic effects of ionizing radiation. By analogy with existing knowledge of spermatogenesis in the mouse, it is probable that no DNA synthesis takes place during this stage. Spermatogonia, in which DNA synthesis does take place, are much more resistant than mature sperm to the action of most mutagens, including X rays. When adult *Drosophila* ♂♂ are treated, mutation frequencies usually drop drastically after a certain time, when germ cells treated as spermatogonia become available for fertilization. Recently, two exceptions have been found among mutagens with alkylating ability. One is 2-chloroethyl methanesulfonate, the other is an ethylenimine derivative of benzoquinone. Both of these produce high mutation frequencies in late broods from treated ♂♂. This is particularly striking in the case of the sulfonate, which produces only few mutations in early broods. In part, this may be due to slow action of the treatment, for in mutation experiments on *Neurospora* this substance required much longer exposures for full effectiveness than other chemical mutagens. Experiments with *Drosophila* indicate that this is, however, not the only and perhaps not even the main explanation for the tendency of mutations to turn up in late broods. It seems that cell division and high metabolic rate during treatment may be essential for optimal effectiveness.

After having dismissed various speculations as disproved or not proved, I may be allowed, perhaps, to suggest a speculation of my own, which is entirely unproved and very possibly entirely wrong. It stems from the recent finding that the joining together of broken chromosome ends is an energy-requiring process, and that adenosine triphosphate is involved as energy donor. Now it appears that chromosome breaks induced by mustard gas join up less readily than X-ray-induced breaks, and it is tempting to relate this peculiarity to the fact that phosphokinases are highly sensitive to inactivation by mustard gas. Breaks induced by triethylene melamine (TEM) likewise have a tendency to remain unjoined but, as far as I know, the action of TEM on enzymes has not yet been analyzed, so that this fact cannot be taken as either supporting or invalidating my hypothesis. Experimental tests are possible, however.

The question may be raised whether there exist chemical mutagens that may be described as "Treffergifte" "hit poisons." This term was coined by the biophysicist P. Jordan and implies action by discrete chemical microevents that have a fixed probability of occurring whenever a molecule of the chemical comes in contact with a molecule of the responding biological system. For such a chemical, the relationship between dose and frequency of induced mutations would be exponential or, at low doses, practically linear. A linear dose relationship has, indeed, been found for several chemical mutagens, but unfortunately this fact is open to a great variety of chemical interpretations. To me, the most conclusive result seems to be that of an experiment in which dose-effect curves after mustard gas treatment were obtained simultaneously for two different genetic effects: lethal mutations due to single spots of chromosome damage, and translocations that arise when two chromosomes have been broken in the same nucleus and subsequently have exchanged pieces. After X radiation, the frequency of translocations increases with dose approximately as the square of the frequency of lethals, as is to be expected if a lethal is the result of a single "hit," while a translocation requires two independent breaks, due to two independent "hits." After mustard gas treatment of *Drosophila* ♂♂, translocation frequency increased with dose almost exactly as the square of lethal frequency. Although more elaborate interpretations of this result are possible, the simplest certainly is that mustard gas induces translocations by two independent events of a kind that singly produce a lethal; that is, that mustard gas acts as a "hit" poison. Similar results have been obtained with TEM and may be similarly interpreted. From the available evidence it is difficult to assess the narrowness of localization of the effects of each hit. Lethals produced by TEM, diepoxybutane, or formaldehyde are more frequently due to small cytologically visible deficiencies than are X-ray-induced lethals; this suggests that some degree of diffusion along the chromosome may take place. On the other hand, the number of cases in which only one of two closely neighboring genes mutates does not appear to be less frequent after chemical treatment than after irradiation.

From its beginning, research on chemical mutagenesis was in large part directed toward the detection of differences from radiation mutagenesis. At first sight, the similarities between the genetic effects of X rays and mustard gas—and also of other chemicals—were much more striking than their differences. All types of genetic effect produced by X rays can be produced by chemical mutagens. Visible mutations after treatment with mustard gas differ from X-ray mutations neither in their types nor in their frequency relative to that of lethal mutation. Pretreatment with infrared light sensitizes the chromosomes to X rays, mustard gas, and nitrogen mustard, the only mutagens that have been tested in this way. Chromosome breaks induced by mustard gas join as freely with X-ray-induced breaks as X-ray-induced breaks join with one another. This, incidentally, is of some relevance to cancer therapy. Although chromosome breakage as such is probably the major cause of the cytostatic action of radiation and radiomimetic poisons, the formation of inviable reunions between broken chromosomes also plays an important role. As far as the production of chromosome breaks is concerned, combination treatment with nitrogen mustard and X rays is additive but, since the number of inviable reunions between two chromosome breaks increases as the square of the number of available breaks, the final cytostatic effect may well be more than additive.

From the start it was realized that it would not be wise to overestimate the significance of the similarity between the genetic effects of X rays and mustard gas. In part, this similarity is due to the use of techniques developed for the detection of just those effects that X rays are known to produce; in part, it is due to the nature of the genetic material that allows only a limited number of observable reactions. It is probable that very different primary mutagenic events may result in indistinguishable final effects. Indeed, some pecularities of mustard gas were detected at a very early stage of the work. The most important of these is the tendency to produce delayed effects; the majority of mustard-gas-induced mutations or chromosome breaks occur first as labile spots on the chromosomes that subsequently, often after many cell divisions, may give rise to a mutation or chromosome break. Alternatively, chromosomes may revert to their original, normal condition. Delayed effect has been proved also for TEM, diepoxybutane, and formaldehyde, and has been revealed as probable for a number of other chemical mutagens. The various interpretations offered are all based on the consideration that the energy provided by a chemical reaction is greatly inferior to that provided by an ionization.

It was obvious from the start also that, after treatment with mustard gas, the various types of genetic effect did not appear in the same relative frequencies as they did after irradiation. In particular, after mustard gas treatment there is a shortage of chromosomal rearrangements in relation to the frequency of lethal mutations. The same result has been found for most chemical mutagens analyzed from this point of view. It is probable that this peculiarity is in large part a consequence of delayed action; if two potential breaks do not open in the same cell cycle, they cannot form a rearrangement. Various mutagens differ vastly, however, in their ability to produce chromosome rearrangements. It would be interesting to see whether they vary concomitantly in their tendency to produce delayed effects, but this has not yet been tested. Some chemical mutagens are nearly as efficient as X rays in the production of chromosome rearrangements; a few may be even more efficient. Others produce hardly any rearrangements. There is no discernible relationship between the ability to produce rearrangements and the chemical class to which a mutagen belongs. All gradations are found among alkylating agents; among purines one compound produces in barley almost exclusively mutations, while the other produces chiefly chromosome rearrangements. This emphasizes once more the point I made at the beginning, namely, that we have at present no evidence for a correlation between chemical class and genetic effects.

That chemical mutagens may single out certain chromosome regions for breakage was first shown for nitrogen mustard and has since been confirmed for a number of other chemicals. Undoubtedly the most exciting possibility of research on chemical mutagens is that it may lead to the detection of substances that single out specific *genes* for mutation. This would not only be of the highest theoretical importance, it would also open the way for controlled manipulation of genotypes. The search for specific gene mutations has been attacked in two ways. The first consists of determining the frequencies with which different mutagens produce certain arbitrarily chosen known mutations. This method has the advantage of a high degree of objectivity. It is, however, not suitable for the detection of mutations that are so highly specific for the chemical under test that they have not occurred before in laboratory stocks. The second method is designed to detect just these mutations. It consists of scoring *all* visible mutations produced by various physical and chemical mutagens and of keeping for further study those that appear to the observer to be new and unusual. The disadvantages of this method are twofold: it depends to an unavoidably large extent on personal judgment, and its results are only qualitative and must be checked and put on a quantitative basis by exceedingly laborious tests. Thus far the second method has been applied to *Antirrhinum* and to *Drosophila*. No details have yet been published on the results with *Antirrhinum*. The *Drosophila* experiments have reached the stage where a number of possibly new mutants are available for testing.

Meanwhile, incontrovertible proof for specificity of mutagenic action has been obtained by the first method applied to microorganisms. In several experiments on bacteria and molds, it could be shown that, of two genes in the same nucleus, one responded preferentially to one mutagen, the second to another. I doubt that the seat of this specificity actually resides in the reaction between gene and mutagen, that is, in the actual process of mutation. What we observe is in no case the mutation itself, but its result, namely, a population of mutated cells. The mutations studied in these experiments were reverse biochemical mutations, that is, mutations that restored to a biochemically deficient strain the ability to synthesize a given growth factor; for example, histidine or adenine. The cell in which such a reverse mutation has occurred must put in motion the whole biochemical machinery for the production of, say, adenine, which an already established gene finds at its disposal in the cell. It seems at least possible that the same chemical treatment that produces mutations interferes also with these post-mutational processes, and that, depending on the chemical, some genes enjoy a better chance than others of becoming established in a line of mutated cells. This would simulate a gene-specific mutation. Experiments to test this possibility are feasible and will, I hope, soon be done. Whatever their results may be, the finding of specific mutagenic effects of chemicals is a very important step forward in mutation work, and further research into specific mutations will certainly rank high among projects in this field.

Discussion

O. G. Fahmy and Myrtle J. Fahmy* (*Chester Beatty Research Institute, Royal Marsden Hospital, London, England*): The classic theory of the mechanism of gene mutation is that the process is random and indeterminate. A mutation, whether spontaneous or radiation-induced, was visualized as arising from an accidental encounter, on the molecular level, between the gene and some energy-conveying particle: electron, proton, or photon (Muller, 1955). It was assumed further that an encounter of the correct type to produce a mutation in a given locus would suffice also to mutate any other locus (Muller, 1952). This simple "model" of the mutation process seemed to be compatible with a great many data of radiation mutagenesis, and it has indeed helped in the development of many quantitative aspects in this field. Recent work, however, particularly on microorganisms, has not supported the principle of random mutability. The work of Demerec (1955) on *Escherichia coli*, and of Giles (1951), Kölmark and Westergaard (1953), and of Kölmark (1953) on *Neurospora* has shown clearly that the mutation process depends on the gene locus and the mutagen. Of particular interest are Kölmark's (1953) results on a double mutant strain of *Neurospora:* adenineless, inositolless. Diepoxybutane proved to be far more effective in mutating the adenine locus.

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By far the most extensive data for differential mutagenicity came from the work on the comparative mutagenic effects of the alkylating compounds and X radiation on *Drosophila melanogaster* (Fahmy and Fahmy, 1956a). Differential response of the genetic material to different mutagens was manifest in the following phenomena: (1) the relative mutation yield of the cell stages during spermatogenesis; (2) the frequency, properties, and distribution of chromosome breaks; (3) the mechanism of induction and distribution of the recessive lethal mutations; (4) the proportion of the various mutations (for example, "visibles" to lethals) induced in the same sample of treated chromosomes; (5) the morphologic expression and distribution of the visible loci induced; and (6) the mutation rates at specific gene loci.

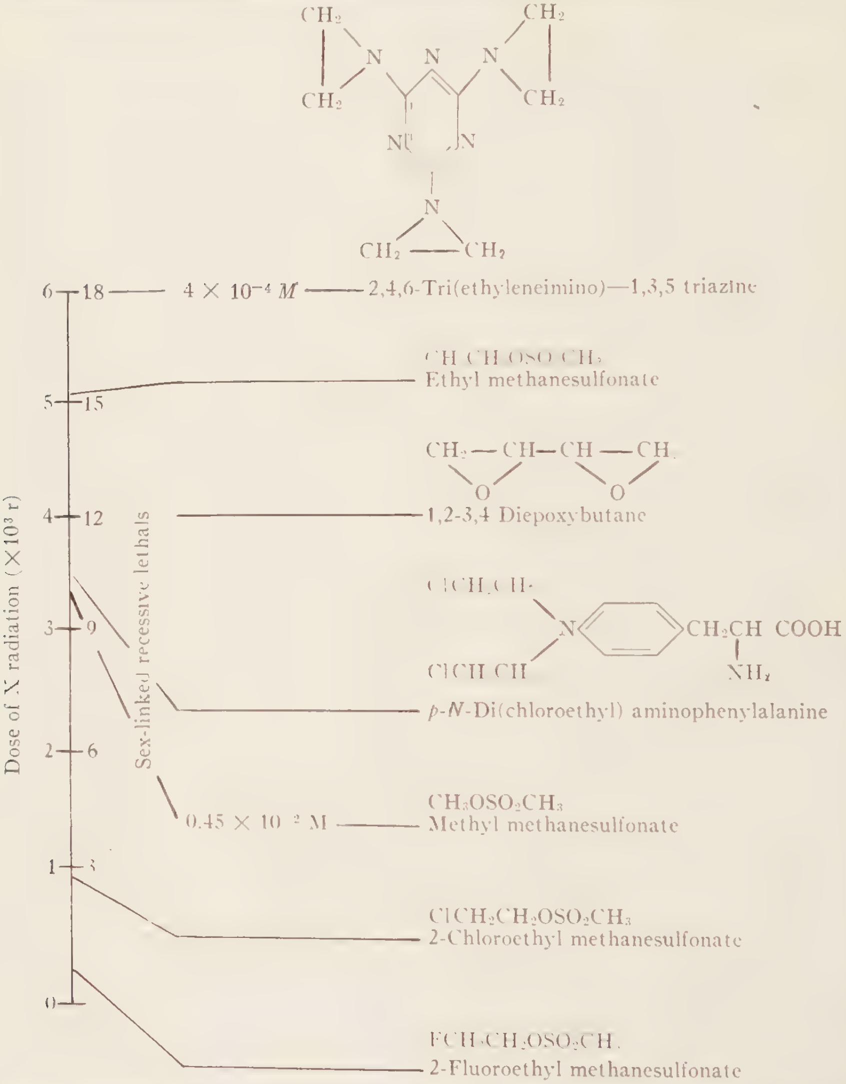
Mutagenicity of the Alkylating Compounds

A general property of the alkylating agents is their evident possession of some degree of mutagenic activity. We have tested about twenty-five of these compounds, belonging to four chemical series (the N-mustards, the epoxides, the polyethylenimines, and the alkyl methanesulfonates). All compounds were dissolved in isotonic saline (0.4 per cent) at the required concentration and were administered by injection around the testes of adult males. Where the compounds were insoluble in water, as in the case of some of the carboxylic and amino acid mustards, the sodium salts were prepared, and these were soluble. To make comparisons between the mutagenicity of different compounds possible, we utilized a standard technique (Fahmy and Fahmy, 1956a) whereby the biological activity of roughly equivalent molar concentrations of the various compounds are tested on postmeiotic germ cells from males of the same genetic strain and of nearly the same age and average weight. The same technique was used also in the irradiation experiments for the determination of the relative mutagenic potency of the chemical and physical agents.

A list has already been published (Fahmy and Fahmy, 1956a) showing the comparative mutagenic efficiency (as regards the induction of sex-linked recessive lethals) for several alkylating compounds in relation to each other and in relation to X radiation. This comparison has revealed that the alkylating agents provide a tool for the induction of mutations as efficient in potency and as wide in range as radiation. More alkylating compounds

TABLE 1

MUTAGENICITY OF SOME ALKYLATING COMPOUNDS* RELATIVE TO X RADIATION ON POSTMEIOTIC STAGES IN ADULT MALES OF *Drosophila melanogaster*



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* Concentration injected is about 1.5×10^{-2} *M*, except where otherwise indicated.

belonging to the mono(methanesulfonoxy) alkanes have since been tested and also proved mutagenic. TABLE 1 shows the mutagenic efficiency of the agents used most extensively in our laboratory for the detailed genetic work; these agents will be frequently referred to in this paper.

Cell Stage Response

When an adult *Drosophila* male was injected with a single dose of a chemical mutagen and then had its germ line fractionated by repeated matings to a succession of virgin females, the mutation rate showed significant fluctuations in the successive broods (Fahmy and Fahmy, 1954; 1955b; 1956b). Since the sperm used in the successive matings must have been derived from progressively younger germ cells at the time of treatment, the fluctuations in the brood-mutation curve should reflect the relative mutagenic response of the various stages of spermatogenesis to the action of the mutagen.

The testis of an adult *Drosophila* male presents all stages of sperm differentiation from spermatogonia through spermatocytes and spermatids to mature sperm. A diagrammatic representation of these stages is given at the top of FIGURE 1. We have also indicated in this figure the speed of utilization of the various germ cells in our standard brood technique. It must be emphasized, however, that the male germ line presents an uninterrupted spectrum of differentiation, and that any attempt at its fractionation into separate cell stages by repeated matings is bound to be very approximate. There is evidence, however, that under our experimental conditions meiosis occurs among cells recovered as sperm late in the fourth, or early in the fifth, broods; that is, 12 to 15 days after treatment (Fahmy and Fahmy, 1954a; 1956b). It can be safely concluded, therefore, that sperm utilized in the first four broods is derived predominantly from postmeiotic germ cells, whereas that utilized in later broods is derived from meiotic and premeiotic stages.

The variation in the yield of the sex-linked recessive lethals in the successive broods under the effect of various alkylating agents is represented graphically in FIGURE 1. The curves illustrated are representative for the various compounds and have been confirmed repeatedly in several experiments. For the stronger mutagens we have chosen experiments with doses that yielded mutation rates of the order of 10 per cent for the most responsive cell stages. This was to guard against complications due to cell killing at higher doses, and to make the comparison valid for mutagenically equivalent levels of the various compounds. The graphs in this figure show most convincingly that the relative mutagenic response of the stages of spermatogenesis to the action of different alkylating compounds was grossly different.

An important point we endeavored to settle in relation to brood-mutation patterns is the extent to which the observed fluctuations were a function of the relative sensitivity of the genetic material during differentiation. On a priori grounds, differences in the physicochemical properties of the various compounds, such as penetrance, stability, and chemical reactivity, might play a role. To test this point, we analyzed the brood-mutation patterns of seven alkyl methanesulfonates that were both monofunctional and difunctional as regards the sulfonoxy groups and that varied also with regard to the structure of the alkyl groups. These compounds varied markedly in chemical reactivity (as measured by the hydrolysis rate) and gave different mutation rates in the sperm. Nevertheless, all seven compounds gave roughly the same brood-mutation pattern, being most active on the sperm and giving a proportionally lower mutation rate for the earlier germ cells. Essentially the same results were obtained with the amino acid mustards. Different compounds that varied in their molecular size and structure, as well as in their chemical reactivity and mutagenic effect, gave roughly the same characteristic brood-mutation pattern. Therefore, it would seem that, for the alkylating agents, penetrance does not play a major role in determining the brood-mutation pattern. This is either

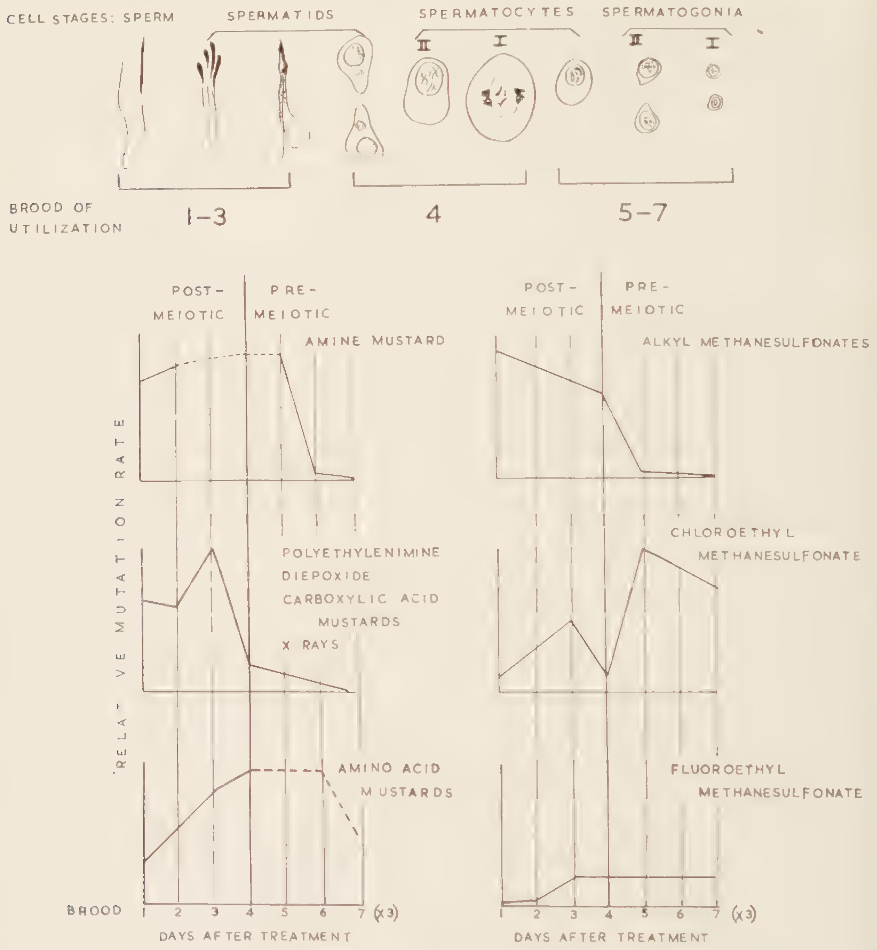


FIGURE 1. Mutation rate during spermatogenesis (sex-linked recessive lethals).

because the membranes and cytoplasm around the genetic material do not provide a barrier against these compounds, or because they do so to roughly the same degree for the various male germ cells. It would follow, therefore, that the brood-mutation pattern is characteristic for each series of the alkylating compounds and is predominantly a function of the sensitivity of the genetic material during sperm differentiation.

In view of the above conclusion, it is of the greatest interest that the chloroethyl and fluoroethyl esters of methanesulfonic acid gave brood patterns grossly different from the other alkyl methanesulfonates. Evidence is available for the belief that this difference may be due, at least in part, to the production of secondary mutagens *in vivo* (Fahmy and Fahmy, 1957c). In the case of chloroethyl methanesulfonate, the secondary mutagen is most probably a highly reactive amino acid mustard.

Chromosome Breaks

It has long been recognized that the rate of major chromosome rearrangements recovered in viable sperm, as well as among recessive lethals, is lower for chemical mutagens as compared to mutagenically equivalent doses—as regards recessive lethals—of radiation (Auerbach and Robson, 1947; Bird and Fahmy, 1953). The reason for this, however, was entirely unknown, since the scoring of the aberrations was undertaken many cell generations after the treatment, and nothing was known about their behavior and fate during the intervening period. A method was needed, therefore, for estimating the primary chromosome breaks directly induced by the chemical treatment. This was achieved through a quantitative study of the yield of "true dominant lethals" with the absolute molar dose of the injected compound. For the determination of the dominant lethal rate the unhatched eggs in the progeny of the treated males were prepared cytologically; only eggs with anomalous chromosome formations were scored as lethal zygotes. Eggs unhatched because of a lack of fertilization were excluded.

The first compound to be analyzed for the induction of dominant lethals was 2,4,6-tri(ethylenimino)-1,3,5-triazine (Fahmy and Fahmy, 1954). Direct cytological analysis of the unhatched eggs showed that they were practically all fertilized. Dominant lethality was due to noneucentric chromosome formations, dicentric and polycentric rearrangements as well as acentric fragments. It was possible also to ascertain that the yield of dominant lethals with the dose of the injected compound (on molar basis) followed exactly the same curve as for X radiation. When the two curves were drawn to the same scale, and for mutagenically equivalent doses (as regards sex-linked recessive lethals), that for the chemical agent was slightly higher, suggesting its higher potency (see figure 2 in Fahmy and Fahmy, 1954).

In sharp contrast to the imine, an amino-acid mustard [*p*-*N*-di(chloroethyl)aminophenylalanine] induced a very low rate of dominant lethals and a very high rate of unhatchability (FIGURE 2). This situation demonstrates clearly how misleading it is to assess dominant lethals on the basis of unhatchability alone, without subsequent cytological analysis of the lethal zygotes. On the basis of unhatchability it would have been concluded erroneously that the amino acid mustard was more effective than X rays in the induction of dominant lethals when, in fact, it was very much weaker in this respect. It is to be noted also that, with the amino acid mustard, cell damage in the testis is predominantly physiological (through cytotoxicity, rather than through the induction of chromosome breakage). Most of the sperm of the treated father must have been killed by the compound, thus causing the eggs of the inseminated mother to be laid unfertilized.

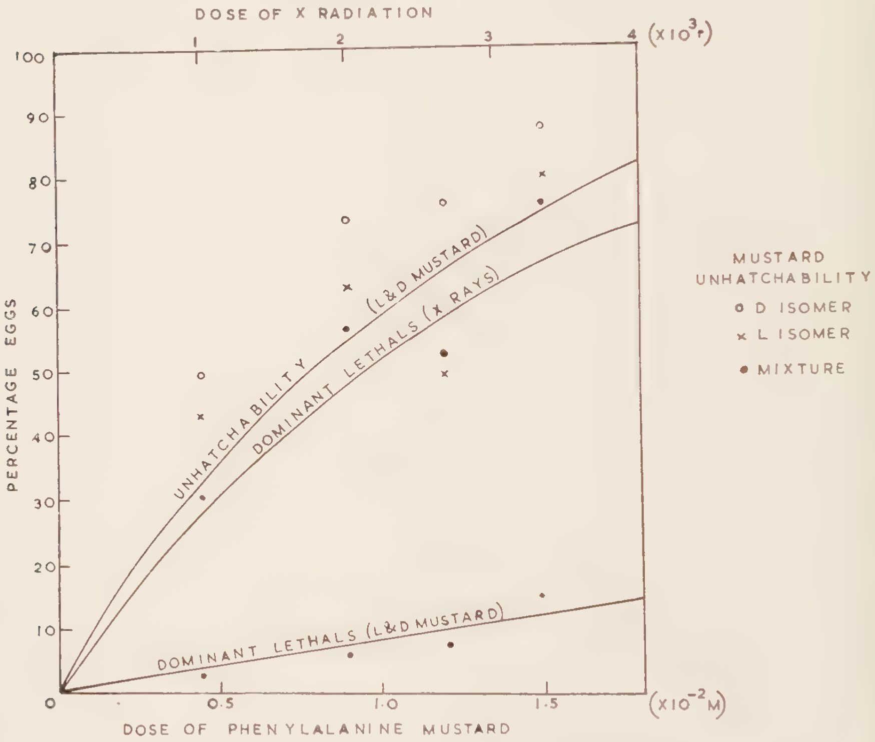


FIGURE 2. Unhatchability and dominant lethals for phenylalanine mustard and X rays.

It was possible to apply the mathematical theory of chromosome rearrangements (Haldane and Lea, 1947) to the dose curve for the dominant lethals induced by a given alkylating agent (Fahmy and Fahmy, 1954). This enabled the translation of the yield of these mutations in terms of the primary chromosome breaks produced per sperm per unit dose, and the probabilities that a break exchanges viably, or inviably. These data have now been calculated for the above imine and amino acid mustard (see table 1 in Fahmy and Fahmy, 1957a) and show some marked differences. For mutagenically equivalent doses, with regard to the induction of sex-linked recessive lethals, the imine is as effective as X rays in the induction of primary chromosome breaks, whereas the efficiency of the amino acid mustard in this respect is only one eighth that of X rays. The properties of the chromosome breaks themselves also differ. The imine breaks tend to undergo more sister union and other inviable rearrangements than to exchange viably, whereas those of the amino acid mustard and X rays show the opposite tendency.

The alkylating agents induce in the chromosomes of the treated sperm, other than the primary effective breaks, a great many potential breaks that open and exchange during the development of the offspring. The frequency of these potential breaks varies with the compound but, on the whole, it is as high as, if not higher than, that for the effective breaks. For the polyfunctional imine, it was possible to estimate that the proportion of potential breaks was 60 per cent of the total initial loci of chromosome damage induced by the treatment (Fahmy and Fahmy, 1955a).

Most important in relation to differential mutagenicity was the fact that the loci of induction of chromosome breaks depended on the mutagen. It has been shown that the distributions of the viable breaks, as detected in the salivary X chromosome of the F_1 larvae, under the effect of tri(ethylenimino)triazine and X rays were grossly different (Fahmy and Fahmy, 1956a).

Recessive Lethals

Detailed cytological analysis of the sex-linked recessive lethals induced by representative examples of the alkylating agents has revealed some general features as to their genetic nature. They all show a shortage of major chromosome rearrangements and a higher frequency of small deficiencies than similar mutations induced at the same rate by X radiation (Bird and Fahmy, 1953; Fahmy and Bird, 1953; Fahmy and Fahmy, 1956a). The rate of rearrangements among recessive lethals is by no means a measure of the initial rate of chromosome breakage induced by the treatment. The shortage of rearrangement lethals for tri(ethylenimino)triazine as compared to X rays, for example, has been shown to be due to differences in the properties of the breaks induced by the two agents, rather than to a lower breakage rate under the effect of the chemical compound (Fahmy and Fahmy, 1956a). On the other hand, small deficiencies do not result in dominant lethals, and those induced by various mutagens are unlikely to be selected differentially against during embryogeny. It would follow, therefore, that the rate of deficiencies observed among recessive lethals is a fairly genuine measure of the rate initially induced in the treated chromosomes.

We have undertaken a comparison of the efficiency of mutagenically equivalent doses of three alkylating agents (the polyethylenimine and two amino acid mustards) as well as X rays, with regard to the induction of both primary chromosome breaks and small deficiencies (Fahmy and Fahmy, 1957a). The primary chromosome breaks for the various agents were estimated from the dominant lethal data. Deficiencies were observed in the salivary gland chromosomes of females heterozygous for sex-linked recessive lethals, and were estimated for the treated chromosomes on the basis of the over-all mutation rate in the sample analyzed. The above comparison has revealed (see table 2, Fahmy and Fahmy, 1957a) that the alkylating compounds induce a rate of small deficiencies nearly double that of mutagenically equivalent doses (as regards the over-all lethal rate) of X radiation. Still more important is the fact that the rate of deficiencies for the chemical agents was independent of the agent's efficiency in the induction of chromosome breaks. This is yet another source of independent evidence (Bird and Fahmy, 1953; Fahmy and Fahmy, 1955b) that small deficiencies induced by chemical agents are caused by failure in gene reproduction *in situ*, and not through chromosome breakage. This is a different mechanism than that postulated for radiation, where it is believed that small deficiencies arise through the breakage of the chromosome at two juxtaposed points by the passage of a single ionizing particle, followed by the reunion of the breakage surfaces (Catcheside, 1948).

Chemically induced recessive lethals differ not only from the comparable radiation mutants in some aspects of their genetic nature and mechanism of induction, but also differ with regard to their loci of induction on the treated chromosome. This became apparent from the study of the distribution of the genetically placed, nonaberration, sex-linked recessive lethals induced by various alkylating compounds as compared to X radiation. Genetic localization was undertaken with 567 of these lethals induced by 3 alkylating compounds, as well as with 70 induced by X rays. The same marker chromosome carrying *scute* (*sc*) *cut* (*ct*) *vermilion* (*v*) *forked* (*f*) *carnation* (*car*) was utilized in all experiments, and the frequencies of the lethals induced between the markers by the different agents were pooled and compared statistically in a contingency table (see tables 3 and 4, Fahmy and Fahmy, 1957b). This very "coarse" pooling was adopted to make the compared distributions independent of all intrasegmental errors in the placing of the individual lethals. In spite of the drastic pooling, a significant difference was discernible between the distributions of the chemically and radiation-induced mutations. This indicates conclusively a differential response of the various segments of the X chromosome to the action of the two classes of mutagens.

"Visible" Mutations

The clearest evidence for differential mutagenicity came from the study of "visible" (morphologically detectable) mutations. The ratio of visibles to lethals induced in the same sample of treated X chromosomes varied considerably, not only between radiation and chemical mutagens, but also among different chemical series of the alkylating agents themselves. The range of the ratios for the different alkylating compounds is given in TABLE 2. Most of the alkylating compounds (Fahmy and Fahmy, 1956a) induced (like the imine, TABLE 2) 10 visible to 100 lethal mutations. This ratio rose to 25:100 for the mono(methanesulfonoxy)alkanes, to 30:100 for the amino acid mustards, and reached a value as high as 40:100 for chloroethyl methanesulfonate. The data on which the above ratios are based were compared in a contingency table and the differences proved to be grossly significant.

The extent to which the differences in the above ratios could have been affected by spurious factors, particularly germinal selection, perhaps deserves further elaboration. The lethals and visibles for most of the alkylating agents are induced in postmeiotic germ cells, where there is no differential selection against the lethals. The amino acid mustards, however, do induce some mutations in premeiotic germ cells, but the ratio of visibles to lethals recovered from these cells was lower (although insignificantly so) than for the mutations recovered from the postmeiotic stages. With chloroethyl methanesulfonate there is a considerable yield of mutations in the premeiotic germ cells and a higher ratio of visibles to lethals than for the postmeiotic stages. The actual data (see table 1 in Fahmy and Fahmy, 1956b) for the premeiotic stages (broods 5 to 7) were 79 visibles to 167 lethals (ratio 0.45) as compared to 33 visibles to 102 lethals (ratio 0.35) for the postmeiotic stages (broods 1 to 4). However, when these data were compared in a fourfold table, the two ratios did not prove significantly different ($\chi^2 = 2.47$; D.F. = 1; P = 0.17). Therefore, with this compound also there was no drastic differential germinal selection against the recessive lethals as compared to the visibles on the X chromosome. We, therefore, pooled the mutations recovered from the premeiotic and postmeiotic germ cells under the effect of the chloroethyl ester and took the over-all ratio as representative. It can thus be concluded that the gross difference in the ratios of visibles to lethals illustrated in TABLE 2 is independent of germinal selection and is a genuine manifestation of the differential response of the genetic material to different chemical series of the alkylating agents.

TABLE 2
RATIO OF SEX-LINKED RECESSIVE VISIBLES TO LETHALS INDUCED BY SOME ALKYLATING AGENTS

Mutagens	No. visibles v	No. lethals l	Total v + l	Ratio v/l	χ^2
Tri(ethylenimino)triazine	109	1008	1117	0.11	69.82
Mono(methanesulfonoxy)alkanes	148	579	727	0.26	0.20
Amino acid mustards	400	1278	1678	0.31	18.13
Chloroethyl methanesulfonate	112	269	381	0.42	22.62
Total	769	3134	3903	0.25	110.77

By far the most interesting feature of the mutagenic action of the alkylating agents is their capacity to induce many "new" visible mutations, in addition to those predominantly encountered in radiation experiments. The morphologic expression of the new mutants does not seem to be confined to any particular part of the external features of the flies. An important characteristic of these mutations, however, is that the majority seem to be pleiotropic, affecting several different parts of the external features simultaneously. Many of these mutants also affect the speed of development of the whole fly and are, therefore, late hatching. Although a great many of these mutants are very clear phenotypically, they are often associated with infertility or even with reduced viability. Great care has been given to the chemically induced mutants that show any phenotypic resemblance to the radiation mutants, and tests of allelism have been undertaken wherever possible.

A few hundred visibles induced by the alkylating agents on the X chromosome have so far been accumulated and placed genetically. Their percentage distribution along the chromosome has been compared with that for the same mutations induced by other mutagens, mainly radiation (Fahmy and Fahmy, 1957a). It was apparent that the radiation visibles predominated in the distal part of the chromosome (0 to 39 crossover units), whereas the chemical visibles predominated in the proximal segment (39 to centromere). To test the statistical significance of this difference in distribution, we compared the relative frequency of the loci induced in the above two segments under the effect of the chemical agents and radiation in a fourfold contingency table (TABLE 3). The difference in this distribution proved to be highly significant ($\chi^2 = 8.00$; D.F. = 1; P = .004).

TABLE 3
DISTRIBUTION OF SEX-LINKED VISIBLES INDUCED BY DIFFERENT MUTAGENS

Mutagen	Genetic map 0 to 38.9	 39 to centromere	Total
Alkylating compounds	155	124	279
Other mutagens (mainly radiation)	105	46	151
Total	260	170	430

Differences in distribution: $\chi^2 = 8.00$; D.F. = 1; P = 0.004.

The occurrence of such a difference in the gross comparison undertaken is very meaningful. The comparison was independent of the acuity of the observer; whether keen or not, he was unlikely to detect (phenotypically) more mutants in one segment of the chromosome than in the other. It was also independent of the quality of the mutant since, in this comparison, it serves only as a marker on the chromosome. It is also independent of the accuracy of the genetic placing because of the very gross pooling. Above all, this comparison surmounts the difficulty as to the occurrence of alleles among the chemical and radiation mutants. If such common alleles occur in large numbers in the two samples, this would bias the comparison toward similarity. The observed gross difference proves conclusively that the alkylating compounds must be producing an appreciable number of "new" visibles and that they are predominantly mutating loci in the proximal part of the X chromosome.

The most direct attack on the problem of differential mutagenicity undertaken in our laboratory consists of tests of mutability at specific visible loci under the effect of radiation and chemical agents. The technique adopted is that of treating the male with the mutagen and then mating it to females carrying the genes under test and scoring the F_1 daughters for the mutations induced at the markers. The loci selected were those suitable for this technique and those that mutated most frequently under the influence of the alkylating agents. For control purposes we also tested some loci with "known" mutability rates under X rays. Of the 17 chemically mutated visibles, only 1 gave intragenic mutations with X rays and at a high rate (8.4×10^{-8} per r); 8 others were eliminated in deletions, but were not mutated intragenically; the remaining 8 were stable to radiation within samples of the size utilized (30,000 to 80,000 tested chromosomes). In contrast, in our experiments the control loci mutated at roughly the same rates as ascertained by earlier workers (Fahmy and Fahmy, 1957b). These results clearly indicated that some of the visible loci responsive to the alkylating agents are less mutable, or possibly even refractile, to radiation. To determine the degree of this differential response at the loci examined, we are now testing the mutation rate for each locus under the effect of the mustard derivative of phenylalanine and by the same technique utilized with radiation. The full comparative results and their statistical analysis will be published in due course. However, the results thus far obtained strongly suggest an appreciable measure of differential response.

Summary

The alkylating agents provide a tool for the induction of mutations as efficient in potency and as wide in range as radiation. The relative mutagenic response during spermatogenesis varies under the effect of different compounds, but is sometimes characteristic of the chemical series. The nature of the mutations induced vary with the agent. A polyfunctional ethylenimine is as effective in producing chromosome breaks as X rays, while the mustard derivatives of amino acids are weak breakers. The alkylating agents induced twice the frequency of small deficiencies as mutagenically equivalent doses of radiation, and this property was independent of the efficiency of the chemical agent in inducing chromosome breaks. The distribution of sex-linked recessive lethals induced by the alkylating compounds is different from that for the same mutations induced by X rays. Many of the visible mutations induced chemically are different in phenotypic expression and genetic position from those induced by radiation. The test of mutability at specific visible loci support the principle of differential gene response to various mutagens.

References

Auerbach, C. & J. M. Robson. 1947. The production of mutations by chemical substances. Proc. Roy. Soc. Edinburgh. **B62**: 271.

Bird, M. J. & O. G. Fahmy. 1953. Cytogenetic analysis of the action of carcinogens and tumour inhibitors in *Drosophila melanogaster*. I. 1:2,3:4-Diepoxybutane. Proc. Roy. Soc. London. **B140**: 556.

Catcheside, D. G. 1948. Genetic effects of radiations. Advances in Genet. **2**: 271.

Demerec, M. 1955. What is a gene? Twenty years later. Am. Naturalist. **89**: 5.

Fahmy, O. G. & M. J. Bird. 1953. Chromosome breaks among recessive lethals induced by chemical mutagens in *Drosophila melanogaster*. Heredity. **6** (Suppl.): 149.

Fahmy, O. G. & M. J. Fahmy. 1954. Cytogenetic analysis of the action of carcinogens and tumour inhibitors in *Drosophila melanogaster*. II. The mechanism of induction of dominant lethals by 2:4:6-tri(ethyleneimino)-1:3:5-triazine. J. Genet. **52**: 603.

Fahmy, O. G. & M. J. Fahmy. 1955a. Cytogenetic analysis of the action of carcinogens and tumour inhibitors in *Drosophila melanogaster*. III. Chromosome structural changes induced by 2:4:6-tri(ethyleneimino)-1:3:5-triazine. J. Genet. **53**: 181.

Fahmy, O. G. & M. J. Fahmy. 1955b. Cytogenetic analysis of the action of carcinogens and tumour inhibitors in *Drosophila melanogaster*. IV. The cell stage during spermatogenesis and the induction of intra- and intergenic mutations by 2:4:6-tri(ethyleneimino)-1:3:5-triazine. J. Genet. **53**: 563.

Fahmy, O. G. & M. J. Fahmy. 1956a. Cytogenetic analysis of the action of carcinogens and tumour inhibitors in *Drosophila melanogaster*. V. Differential genetic response to the alkylating mutagens and X-radiation. J. Genet. **54**: 146.

Fahmy, O. G. & M. J. Fahmy. 1956b. Mutagenicity of 2 chloroethyl methanesulphonate in *Drosophila melanogaster*. Nature. **177**: 996.

Fahmy, O. G. & M. J. Fahmy. 1957a. Comparison of chemically and X-ray induced mutations in *Drosophila melanogaster*. Advances in Radiobiology. : 437. Oliver & Boyd. Edinburgh, Scotland.

Fahmy, O. G. & M. J. Fahmy. 1957b. Further evidence for differential effects of mutagens in *Drosophila melanogaster*. J. Genet. **55**: 280.

Fahmy, O. G. & M. J. Fahmy. 1957c. Mutagenic response to the alkyl methanesulphonates during spermatogenesis in *Drosophila melanogaster*. Nature. **180**: 31.

Giles, N. H., Jr. 1951. Studies on the mechanism of reversion in biochemical mutants of *Neurospora crassa*. Cold Spring Harbor Symposia Quant. Biol. **16**: 283.

Haldane, J. B. S. & D. E. Lea. 1947. A mathematical theory of chromosomal rearrangements. J. Genet. **48**: 1.

Kölmark, G. 1953. Differential response to mutagens as studied by the *Neurospora* reversion mutation test. Hereditas. **39**: 271.

Kölmark, G. & M. Westergaard. 1953. Further studies on chemically induced reversions at the adenine locus of *Neurospora*. Hereditas. **39**: 209.

Muller, H. J. 1952. Gene mutations caused by radiation. *In* Symposium on Radiobiology. Natl. Acad. Sci. Wiley & Sons. New York, N. Y.

Muller, H. J. 1955. How radiation changes the genetic constitution. Bull. Atomic Scientists. **11**: 329.

Irwin I. Oster (*Department of Zoology, Indiana University, Bloomington, Ind.*): I believe that C. Auerbach has summarized very adequately all the findings reported concerning the effects of alkylating agents on the hereditary material. Her major contributions in this field, commencing with her discovery of the mutagenic action of mustard gas, have enabled her to present a rather complete picture of the facts and to offer some interesting suggestions concerning the mode of action of the alkylating agents. A comparison of the effects of these agents with those produced by X rays indicates the existence of some qualitative differences, such as the occurrence of gene mutations in cell generations fairly far removed from the generation that had been treated with these chemical mutagens, and some quantitative differences, such as the relative paucity of gross structural changes of the chromosomes after these chemical treatments as contrasted with their frequency following X-ray treatment. At the present time these differences can be ascribed mainly to the ability of chemical mutagens to produce delayed effects. As a matter of fact, the only apparent difference thus far reported between mutagenesis by X rays and chemicals (that is, the finding that varying the oxygen tension during treatment greatly affects X-ray mutagenesis, but not mustard gas mutagenesis), may be due to the ineffectiveness of a relatively short oxygen exposure in cells acted upon by mutagens that exhibit delayed effects. In addition, it should be pointed out that the ineffectiveness of altering the oxygen tension during mustard gas treatment does not imply a different mode of action for this chemical, especially when one considers such things as the finding that, while a variation of the oxygen tension affects mutagenesis by X rays, it does not affect mutagenesis by neutrons. However, both of these ionizing mutagens probably act via the same mechanisms, the difference observed being due to a difference in ion density rather than ion types.

As for the material presented by the Fahmys, I should mention perhaps that their findings as presented here appear to differ somewhat from those contained in some of their previously published papers. Thus, it would be necessary to have all the data on which they base their new far-reaching conclusions before these can be evaluated adequately. Regarding the failure to obtain the new visible mutations by X rays, I feel that the scale on which the experiments were done was not large enough to lead to more than very tentative inferences. Furthermore, I should point out (although I am sure they are aware of it) that special genetic stocks of the fruit fly, *Drosophila melanogaster*, have been constructed by H. J. Muller and his associates in a way paralleling the degrees of precision and complication of the compounds that have been synthesized by the biochemists, and that these are available for subjecting the Fahmys' findings to further proof and elucidation.

C. AUERBACH: I agree with Oster's last statement. In this connection I should like to point out that not even deficiencies were obtained for a large proportion of the tested mutations. This shows that the scale of the work was not large enough; it can hardly be imagined that the presence of these loci—before mutation—could protect the chromosome region in which they occur against breakage by X rays. Regarding the other claims made by the Fahmys, I admire the tremendous amount of work that must have gone into their formulation but, unfortunately, so few data have been published that it is not possible at present for geneticists to judge of the validity of practically any of the conclusions drawn.

CARCINOGENIC ACTION OF ALKYLATING AGENTS

By Arthur L. Walpole
Pharmaceuticals Division, Imperial Chemical Industries, Ltd., Manchester, England

A few years ago it became increasingly apparent that the biological effects of various sulfur and nitrogen mustards closely resemble those of X rays. Observations upon their local and systemic toxicity—in particular, their hematological effects in normal subjects and, later, in leukemic patients—their mutagenic activity, their inhibitory action upon the growth of experimental tumors, and their cytotoxic effects upon proliferating cells, all pointed to this conclusion. Several of these compounds were therefore tested for carcinogenicity, with the result that the first direct evidence of carcinogenic activity in alkylating agents was obtained.

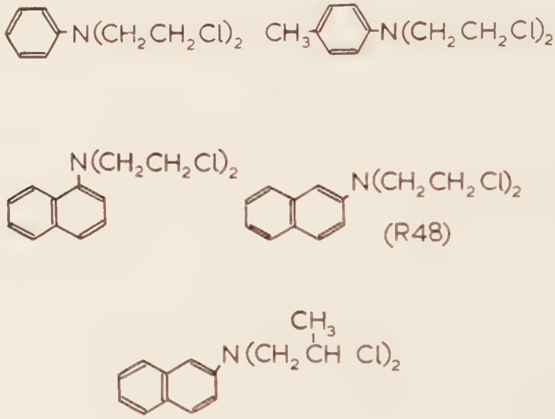


FIGURE 1

Haddow[1] produced tumors in mice, rats, and hamsters with several bifunctional aromatic nitrogen mustards (FIGURE 1) given subcutaneously or by mouth, and later he obtained a similar result from the subcutaneous injection in rats of mustard gas. Independently of this, Boyland and Horning[2] had observed tumors, mainly of the lung, in mice given HN2 or HN3, and Heston[3] observed an increased incidence of pulmonary tumors in strain A mice with HN2. Griffin *et al.*[4-6] also have reported the induction of tumors of several types in rats and mice given these two substances by various routes.

With the discovery of radiomimetic properties—growth inhibitory, mutagenic, and specific cytotoxic activity—in analogous compounds of other series, such as epoxides and ethylenimine derivatives and, later, methanesulfonoxyalkanes, it became of interest to determine whether these, too, might not be carcinogenic. Interest in these substances, as in the mustards, has centered largely on their growth-inhibitory properties and on their potential usefulness in the treatment of malignant disease. Pronounced tumor-inhibitory activity was found here, as in the mustards, to be limited to polyfunctional compounds, although to this generalization a few notable exceptions are known. For this reason, carcinogenic testing was at first confined to polyfunctional members of the newer series, and some evidence of activity in this sense was obtained with several of these.

With a rather crude preparation of vinylcyclohexene dioxide (FIGURE 2) given intraperitoneally to rats, our results were equivocal and, with more purified samples given subcutaneously, the tumor yield was negligible. The impure material, applied repeatedly in acetone to the skin of mice, gave epithelial carcinomata in good yield, but again we failed to obtain a clear-cut result with a purified preparation. In our latest test, in which dosage was low owing to the accidental loss of some material, few mice survived for any length of time and, in these, only one papilloma was detected.

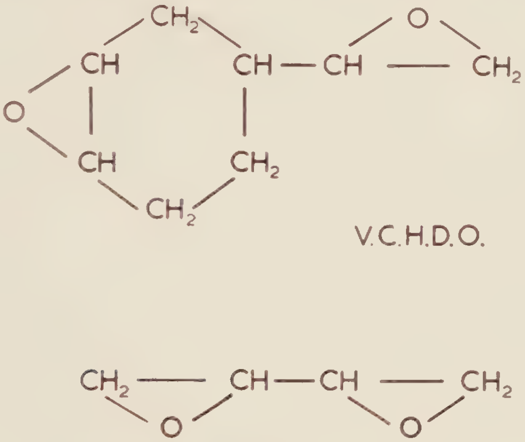


FIGURE 2

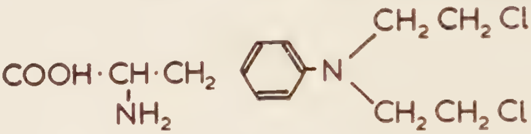
In all our experiments with a second diepoxide, butadiene dioxide (FIGURE 2), mortality among the treated animals, rats and mice, was high, and the incidence of tumors in the survivors was negligible. However, Koller[6] has recorded briefly the production of sarcomata in rats with this substance.

In the ethylenimine series we found that triethylene melamine (TEM), given intravenously, increased the incidence of lung tumors in strain A mice. The same result was observed independently by Shimkin.[7] We failed to induce neoplasia in stock mice by the subcutaneous injection of TEM in aqueous solution, but repeated subcutaneous injection in arachis oil into stock rats, in a total dose of 1 mg. of TEM per 100 gm. of body weight, gave sarcomata in good yield at the site of injection. Such tumors developed in 11 of the 12 rats treated in the comparatively short time of 240 to 450 days after the first dose (the 12th rat died on the 19th day). Repetition of this experiment in inbred Wistar albino rats resulted in local sarcomata later and in lower total yield (tumors appeared in 5 of the 12 rats between 438 and

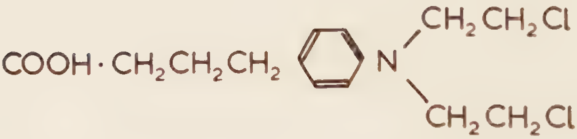
506 days), but 2 of these animals developed hepatoma and 2 developed an ovarian tumor of most unusual type.

We have tested only one other polyfunctional ethylenimine derivative in this manner, the substance being 1,3-bis(ethyleniminosulfonyl)propane (BEP). Six of 12 rats given a total dose of 8 mg. per 100 gm. developed local sarcomata and, although the tumors were rather late in appearing, this result is significant.

Koller[6] has described tumors resulting from the subcutaneous injection in rats of 1,4-dimethanesulfonoxybutane (Myleran), but the details of treatment and tumor incidence have not been published. Shimkin[7] failed to obtain lung tumors in strain A mice given this substance, and Roe and Salaman[8] saw neither lung tumors nor, indeed, tumors of any kind, in skin-painting experiments with the substance in the mouse.



(CB 3007)



(CB 1348)

FIGURE 3

The total process of tumor induction in the mouse skin seems to occur in two stages, "initiation" and "promotion," and corresponding categories of active agent, initiators, and promoters, as well as complete carcinogens, are now recognized. Salaman and Roe[9] have recently examined a number of polyfunctional alkylating agents for activity as initiators or complete carcinogens. For this purpose the agents were applied to the mouse skin either alone or followed by croton oil. This substance is an active promoting agent, but without an initiator it gives rise to very few tumors.

In these tests it was found that bis(2-chloroethyl)-β-naphthylamine (R48) was quite inactive as an initiator, while HN2, and both the D- and L-forms of *p*-bis(2-chloroethyl)aminophenylalanine (CB 3007, FIGURE 3), gave a small and statistically insignificant increase in the yield of skin papillomas. With both forms of this phenylalanine derivative, one mouse eventually developed a malignant epithelioma, and both these isomers increased also the incidence of lung adenomata in the mice. The only mustard tested that gave unequivocal evidence of initiating activity was *p*-bis(2-chloroethyl)aminophenylbutyric acid (CB 1348, or chlorambucil, FIGURE 3).

Under other test conditions, as already mentioned, tumors could be induced both with HN2 and R48. The compound CB 1348 and a racemic mixture of the isomers of the phenylalanine mustard have been tested for carcinogenicity by Haddow,[10] who administered them in arachis oil by subcutaneous injection to rats. CB 1348 was given also in similar manner to mice. The significance of the very small yield of local sarcomata obtained in these experiments is in doubt.

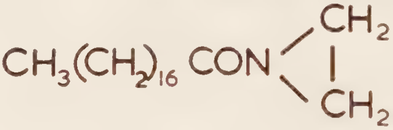
Roe and Salaman[8] have found that TEM does not give rise to skin tumors, nor to epidermal hyperplasia or other recognizable change, when applied repeatedly in maximum sublethal doses, without croton oil, to the skin of mice. On the other hand, a single application of TEM (0.5 mg.), if followed by croton oil, proved sufficient to increase significantly the incidence of papillomas. Thus, for the mouse skin, TEM was an effective initiator of carcinogenesis, but it was not of itself carcinogenic, at least in the doses used.

As already mentioned, Roe and Salaman[8] obtained no tumors by applying Myleran to the skin of mice. This substance proved inactive also as an initiator, but four of a series of related compounds have since been reported (Roe[11]) to have initiating activity. These are the *cis*- and *trans*-isomers of 1,4-dimethanesulfonoxybutene; 1,4-dimethylsulfonoxybutyne; and allyl methanesulfonate.

It is not unusual, of course, for carcinogens to produce different results in different circumstances, and even to appear active or inactive according to the method of test. The response in any instance is governed not only by the intrinsic activity of the substance given and the susceptibility of different tissues to neoplastic change, but also by the physical and chemical properties of the substance as they affect absorption, distribution, and elimination under the particular conditions of test. With the mouse skin, at least, the position is further complicated by the phenomena of initiation and promotion. As yet, there is no proof that these two processes are involved in carcinogenesis at sites other than the skin, but I have not found any evidence that excludes this possibility. In all these factors we have sufficient basis for the variations observed in the response to carcinogens. Whether a tissue may have a peculiar inherent susceptibility to any particular carcinogen independent of such considerations is open to doubt.

Thus, there is no "omnicompetent" test for carcinogenic activity, and negative results obtained by any one procedure have only limited significance. On the other hand, a statistical increase in the incidence of neoplasia in any well-controlled test is conclusive evidence of (intrinsic) carcinogenic activity, even though this may not be demonstrable under other conditions of test. On this criterion it is established beyond doubt that certain polyfunctional sulfur and nitrogen mustards and ethylenimines are carcinogenic, while the evidence for like activity in bisepoxides and dimethanesulfonoxyalkanes is suggestive, at the very least. Having in mind the many factors involved in the response to carcinogens, one can hardly expect to find a simple relationship between structure and demonstrable activity. By the same token, perhaps one should not attach too much importance to the uncertainty of action of some polyfunctional alkylating agents. At the same time, their behavior in this respect is in striking contrast to that of some related monofunctional substances with which we have worked.

The first of these to be examined was stearoylethylenimine (FIGURE 4), although we thought at the time that carcinogenic activity, like tumor-inhibitory activity, would be limited in the main to polyfunctional derivatives. However, these we thought might act by intermolecular condensation within the cell to yield polyreactive polymer units, to which we ascribed the final radiomimetic effect. We felt that a substance such as stearoylethylenimine should form micelles by the association of the long fatty chain in its molecules—micelles that, in form and reactivity, would simulate our hypothetical polymer units. Thus, it seemed possible that, although this substance is monofunctional, it might well behave like a polyfunctional compound of the more conventional kind.



$$CH_3(CH_2)_{16}\,CON\begin{matrix}\diagup CH_2\\ \quad|\\ \diagdown CH_2\end{matrix}$$

FIGURE 4

Repeated subcutaneous injection in rats of stearoylethylenimine in arachis oil resulted in the appearance of sarcomata at the injection site in significant yield. We then tested lower homologues in the acyl ethylenimine series, in the knowledge that, as the fatty chain became progressively shorter, the tendency toward micelle formation would diminish and finally disappear. It soon became apparent that activity is retained throughout the series down to its lowest members, being found, for example, in acetylethylenimine. We also obtained tumors with ethylenimines other than the acyl derivatives, and with ethylenimine itself. From this it appeared that neither polyfunctionality nor cross linkage, nor the formation of a polyreactive polymer or micelle is necessary for carcinogenic action in ethylenimine derivatives, whatever may be their significance for other forms of radiomimetic effect. On the other hand, we encountered a few substances, all alkyl sulfonethylenimines, in which we could detect no activity by this method of test. It accordingly appeared that, although the ethylenimine grouping may itself be responsible for carcinogenic activity, its presence in a derivative is not alone sufficient for a demonstrable effect. Other features of the molecule are also important in that they modulate chemical reactivity and affect physical properties in a favorable or unfavorable sense.

Of these carcinogenic monofunctional ethylenimine derivatives, none that we have so far tested against the Walker tumor in rats significantly inhibits its growth. When given to a rat bearing this tumor (in the largest single dose that allows the animal to survive for 24 hr.), none causes the appearance, in tumor tissue sampled after 24 hr., of the gross chromosomal aberrations -fragmentation and bridge formation at mitotic anaphase--regarded as distinctive of the radiomimetic effect. Thus, although these substances are carcinogenic they provide no exception to the general rule that, for these other manifestations of radiomimetic activity, polyfunctionality is required. On the other hand, I draw attention to an observation made in our laboratories by Ockey,[12] who has shown that, when applied in high concentrations to the root tip of the broad bean, acetylethylenimine causes chromosomal fragmentation and bridge formation comparable in extent with that produced at very much lower concentration by the polyfunctional ethylenimine, TEM. It thus appears that, with respect to the production of these effects, the difference between monofunctional and polyfunctional ethylenimines is quantitative rather than qualitative.

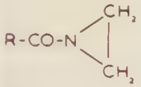
 R =	DOSAGE (MAX) Total mg / 100 g	Duration (days)	LOCAL SARCOMATA Rats affected / 12	Time to appearance (days)
$C_{17}H_{33}$ (OLEYL) —	170	49	11	138 – 178
$CH_3(CH_2)_{16}$ —	110	35	7	254 – 509 (3 <400)
$CH_3(CH_2)_{12}$ —	330	141	8	150 – 229
$CH_3(CH_2)_{10}$ —	100	32	1	522
	12 5	1	1	123
$CH_3(CH_2)_7$ —	80	191	11	121 – 296
$CH_3(CH_2)_4$ —	50	133	12	196 – 314
$(C_2H_5)_2CH$ —	43	198	9	169 – 301
$CH_3(CH_2)_2$ —	22	198	11	177 – 344
CH_3 —	1 5	184	4/6♂	196 – 393
	7 8	184	6/6♀	247 – 288 482

FIGURE 5. Induction of sarcomata at the site of repeated subcutaneous injection in arachis oil of some acyl ethylenimines.

Most of our earlier work with these substances has now been published (Walpole *et al.*[13]), and I include FIGURES 5 and 6 only to illustrate the kind of results we obtained. They refer to sarcomata produced in rats at the injection site by repeated subcutaneous injection of compounds dissolved or dispersed in arachis oil. The high incidence and early appearance of the tumors with many of these compounds are noteworthy and distinguish them from the few late sarcomata often seen with arachis oil alone.

Several acyl ethylenimines of intermediate chain length were given subcutaneously to rats in Carbowax 300, and several of the lower members in aqueous solution. Ethylenimine itself and β-propiolactone were given in both oil and water, and a selection of the acyl derivatives, in oily solution, was also tested in mice. Results comparable to those shown in FIGURES 6 and 7 were obtained in almost every case.

The carcinogenic effect of these substances is not confined, however, to the production of local sarcomata. Already in the experiments referred to, a small but significant incidence of other forms of neoplasia had been seen in rats in several of the treated groups. They included, in particular, mammary carcinoma and carcinoma of the acoustic sebaceous gland. In more recent experiments with nonanoylethylenimine, taken as representative of the acyl ethylenimine series, we have obtained tumors by intravenous injection in rats and skin painting in mice. An oily solution of this substance, emulsified in an aqueous medium and given intravenously to a group of twelve rats, produced carcinoma of the bladder in one and a malignant papilloma of the renal pelvis in one other. The control emulsion produced no more than slight hyperplasia of the epithelium of the renal pelvis in any case.

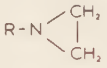
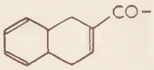
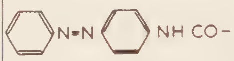
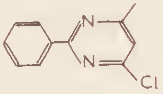
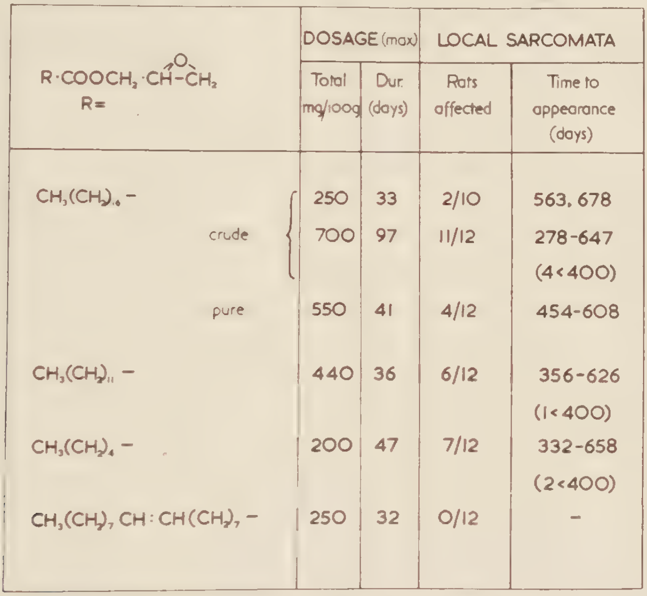
 R =	DOSAGE (max)		LOCAL SARCOMATA	
	Total (mg/100g)	Duration (days)	Rats affected /12	Time to appearance (days)
	ca 110	141	12	169-358
	113	108	7	277-515 (2<400)
	96	114	11	111-154
$CH_3(CH_2)_6SO_2-$	150	54	0	-
$CH_3(CH_2)_4SO_2-$	90	50	0	-
$CH_3(CH_2)_2SO_2-$	125	50	0	-

FIGURE 6. Induction of sarcomata at the site of subcutaneous injection in arachis oil of miscellaneous ethylenimine derivatives.

The result of skin painting in mice seems to depend largely upon the strain of animal used. In C3Hf mice, squamous cell carcinoma in the painted area predominates, while in random-bred mice of the Chester Beatty strain, similarly treated, lymphosarcoma of thymic origin develops in a significant proportion of the animals before the appearance of the first papilloma of the skin. It may be noted in passing that β-propiolactone has been shown to be a powerful initiator of skin carcinogenesis in the mouse. It has since been found[14] that this substance, when applied repeatedly without croton-oil treatment, causes the eventual appearance of a few papillomas that undergo early malignant change.

Limited tests with epoxides have shown that here, too, carcinogenic activity can be demonstrated with some monofunctional derivatives. Results we have obtained with a few such substances are shown in FIGURES 7 and 8. FIGURE 7 relates to a small series of the glycidyl esters of fatty acids. In all cases the vehicle was arachis oil. It is immediately apparent that the doses used here were much higher than those with the ethylenimine derivatives, and that, in general, the tumors developed later. FIGURE 8 refers to some miscellaneous epoxides, including ethylene oxide itself. Here again the same two tendencies may be noted, and with some substances no tumors at all were obtained. One cannot be entirely happy about sarcomata resulting from the presence in the *subcutis* of the rat of such large quantities of unphysiological substances, since hypertonic saline[15] and concentrated aqueous glucose[16] and, indeed, arachis oil itself,[13] have been reported to have a like effect. In one instance, that of the glycidyl ether of p-hydroxydiphenylamine, the yield was high and the tumors appeared early but, in



$R{\cdot}COOCH_2{\cdot}\overset{O}{CH{-}CH_2}$	DOSAGE (max)		LOCAL SARCOMATA	
R=	Total mg/100g	Dur. (days)	Rats affected	Time to appearance (days)
$CH_3(CH_2)_{16}-$ crude	250	33	2/10	563, 678
	700	97	11/12	278-647 (4<400)
pure	550	41	4/12	454-608
$CH_3(CH_2)_{11}-$	440	36	6/12	356-626 (1<400)
$CH_3(CH_2)_4-$	200	47	7/12	332-658 (2<400)
$CH_3(CH_2)_7CH{:}CH(CH_2)_7-$	250	32	0/12	-

FIGURE 7. Induction of sarcomata at the site of subcutaneous injection in arachis oil of some glycidyl esters of fatty acids.

general, further examination of these substances by other methods of testing is needed.

From these observations emerges the broad conclusion that the presence in a molecule of one or more groups having alkylating function may result in carcinogenic activity. Other features of the molecule, however, have a decisive influence upon the level of such activity and upon its manifestation in any given circumstances. These considerations suggest that direct chemical interaction with some cellular component is an essential feature of the carcinogenic process with these agents. There is evidence that, in appropriate circumstances, they all interfere in some way with the normal orderly replication of the chromosomes. Thus, it may well be that their carcinogenic action depends upon some distortion in the normal replication either of the chromosomes themselves or of some other determinative, self-reproducing, cellular component.

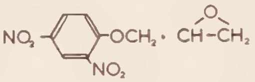
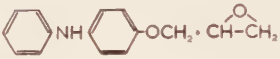
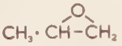
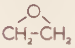
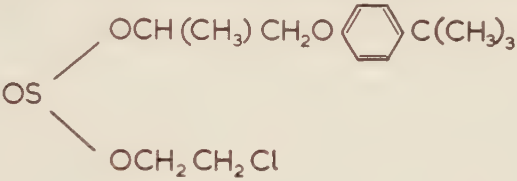
SUBSTANCE	DOSAGE(max)		LOCAL SARCOMATA	
	Total mg/100g	Dur (days)	Rats affected	Time to appearance (days)
	360	105	0/12	–
	475	125	11/12	286–456 6<400
 (in oil)	150	325	8/12	507–739
(in water)	150	325	3/12	158, 737(2)
 (in oil)	100	94	0/12	–

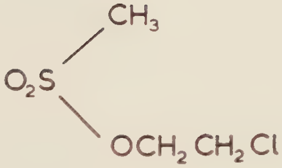
FIGURE 8. Induction of sarcomata at the site of subcutaneous injection in arachis oil or water of miscellaneous epoxides.

I shall not speculate further upon the theoretical implications of any of these observations, but I draw attention to the problem they raise in connection with occupational and public health. The knowledge that certain alkylating agents—methylolamides, epoxides, and ethylenimines—had been used for textile finishing, and that their use for this purpose depended upon cross-linkage effects, played a notable part in directing attention to them as possible radiomimetic agents. There is still interest in the use of these substances for improving the physical properties of textiles and, for example, as waterproofing agents. More recently, interest has developed in dyestuffs that owe their substantivity for fibers, at least in part, to reactive groupings of the kind with which we are here concerned. Meanwhile, the use of bis-epoxides as the basis of industrial polymers has developed enormously, and these substances are finding increasing application in, for example, the paint and varnish industry, in both the monomeric and polymerized states. For this kind of usage no real suspicion attaches to polymers, in which the free epoxide content is negligible. The hazard is probably greatest with monomeric material containing reactive and readily diffusible molecules of low molecular weight.

Another compound not unrelated to those we are considering has recently been used as an insecticide. As long ago as 1929 it was recorded that certain esters of sulfurous acid, among them bis(2-chloroethyl)sulfite, are toxic to grain weevils,[17] and later it was found that related esters are lethal to the spider mite. Following this lead, Harris and Zukel[18] found optimal activity in 2-(*p-tert*-butylphenoxy)isopropyl-2-chloroethyl sulfite, which has been introduced into commerce as a miticide under the name Aramite (FIGURE 9). Tests reported by Lehman[19] of the United States Food and Drug Administration, Washington, D.C., have shown that Aramite produces hepatoma in rats when given in the diet at a level of 0.5 per cent. Particular interest attaches to this substance in view of its chemical relationship to 2-chloroethyl methanesulfonate (CB 1506, FIGURE 9), which inhibits tumor growth[20] and has a distinctive mutagenic action.[21]



"ARAMITE"



CB 1506

FIGURE 9

Finally, I refer to observations of perhaps even wider import. The first is the finding of Fieser and his collaborators[22] that certain oxidation products of cholesterol, including a hydroperoxide, produce sarcomata on subcutaneous injection in sesame oil in mice. The implication is that such substances, formed in the body, may play a part in causing so-called spontaneous cancer. Other pertinent observations are those of Kotin and his co-workers,[23] who have produced skin tumors in mice by painting with acetone solutions of aliphatic air pollutants or oxidation products of aliphatic hydrocarbons, and of Kotin and Falk,[24] who have produced lung tumors in mice by exposure to an atmosphere of ozonized gasoline. In such oxidations, epoxides are among the products formed to which the observed tumor-inductive effect may be due.

From these examples it is evident that the carcinogenic potentiality of alkylating agents is not merely an academic question, but a matter of immediate practical concern. It is essential that we know more about the factors, chemical and physical, that determine the action of individual agents, and about the conditions, if any, under which any one can be safely made or used. For any of these agents evidence of inactivity should be accepted with reserve, and radiomimetic toxic effects, as well as tumor-producing action, should be considered before recommending a substance for widespread use under uncontrolled conditions of exposure. As yet there is no evidence that any alkylating agent has caused tumors in man, but past experience in the industrial handling of heavy hydrocarbons and aromatic amines indicates that chances should not be taken with alkylating agents.

References

1. Haddow, A. 1953. Mechanisms of carcinogenesis. II. Biologic alkylating agents. *In* The Physiopathology of Cancer. : 478. F. Homburger and W. H. Fishman, Eds. Hoeber. New York, N. Y.
2. Boyland, E. & E. S. Horning. 1949. The induction of tumours with nitrogen mustards. Brit. J. Cancer. **3**: 118.
3. Heston, W. E. 1949. Induction of pulmonary tumors in strain A mice with methyl bis(beta-chloroethyl)amine hydrochloride. J. Natl. Cancer Inst. **10**: 125.
4. Griffin, A. C., E. L. Brandt & E. L. Tatum. 1950. Nitrogen mustards as cancer-inducing agents. J. Am. Med. Assoc. **144**: 571.
5. Griffin, A. C., E. L. Brandt & E. L. Tatum. 1950. Induction of tumours with nitrogen mustards. Cancer Research. **11**: 253.
6. Koller, P. 1953. Dicentric chromosomes in a rat tumour induced by an aromatic nitrogen mustard. Heredity. **6** (Suppl.) : 181.
7. Shimkin, M. B. 1954. Pulmonary-tumor induction in mice with chemical agents used in the clinical management of lymphomas. Cancer. **7**: 410.
8. Roe, F. J. C. & M. H. Salaman. 1955. Further studies of incomplete carcinogenesis: triethylene melamine (T. E. M.), 1: 2-benzanthracene and beta-propiolactone, as initiators of skin tumour formation in the mouse. Brit. J. Cancer. **9**: 177.
9. Salaman, M. H. & F. J. C. Roe. 1956. Further tests for tumour initiating activity: N:N-di-(2-chloroethyl)-*p*-amino-phenylbutyric acid (CB. 1348) as an initiator of skin tumour formation in the mouse. Brit. J. Cancer. **10**: 363.
10. Haddow, A. Personal communication.
11. Roe, F. J. C. 1956. Tumor initiation in mouse skin by certain esters of methanesulfonic acid. Cancer Research. **17**: 64.
12. Ockey, C. H. 1958. A quantitative comparison of the cytotoxic effects of proflavine, acetylethyleneimine and triethylene melamine on root-tips of *Vicia faba*. In press.
13. Walpole, A. L., D. C. Roberts, F. L. Rose, J. A. Hendry & R. F. Homer. 1954. Cytotoxic agents. IV. The carcinogenic actions of some monofunctional ethyleneimine derivatives. Brit. J. Pharmacol. **9**: 306.
14. Roe, F. J. C. & O. M. Glendenning. 1956. The carcinogenicity of beta propiolactone for mouse-skin. Brit. J. Cancer. **10**: 357.

15. Tomoro, Y. 1940. Über die artificielle Erzeugung des Sarkoms bei den weissen Ratten mittels konzentrierter Kochsalzlösung. Gann. **34**: 149.
16. Nishiyama, Y. 1938. Experimentelle Erzeugung des Sarkoms bei Ratten durch wiederholte Injektionen von Glucoselösung. Gann. **32**: 85.
17. I. G. Farbenindustrie, A.G. 1929. Brit. Patent 346,685. Ger. Patent 514,496.
18. Harris, W. D. & J. W. Zukel. 1954. Chemical structure of a series of organic sulfites and its toxicity to the two-spotted spider-mite. J. Agr. Food Chem. **2**: 140.
19. Lehman, A. J. 1952. Chemicals in foods: a report to the association of food and drug officials on current developments. Part II. Pesticides. Sect. V. Pathology. Assoc. Food Drug Officials U. S. Quart. Bull. **16**: 126.
20. Haddow, A. & W. C. J. Ross. 1956. Tumour growth-inhibitory alkyl sulphonates. Nature. **177**: 995.
21. Fahmy, O. G. & M. J. Fahmy. 1956. Mutagenicity of 2-chloroethyl methanesulphonate in *Drosophila melanogaster*. Nature. **177**: 996.
22. Fieser, L. F., T. W. Greene, F. Bischoff, G. Lopez & J. J. Rupp. 1955. A carcinogenic oxidation product of cholesterol. J. Am. Chem. Soc. **77**: 3928.
23. Kotin, P., H. L. Falk & M. Thomas. 1956. Production of skin tumors in mice with oxidation products of aliphatic hydrocarbons. Cancer. **9**: 905.
24. Kotin, P. & H. L. Falk. 1956. The experimental induction of pulmonary tumors in strain A mice after their exposure to an atmosphere of ozonized gasoline. Cancer. **9**: 910.

THE COMPARATIVE EFFECTS OF FIVE POLYFUNCTIONAL ALKYLATING AGENTS ON THE RAT FETUS, WITH ADDITIONAL NOTES ON THE CHICK EMBRYO*

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Polyfunctional alkylating agents have been studied for evidence of individual specificity of action on normal tissues and on tumors by using biological systems in which effects on multiple factors can be observed simultaneously. In these systems apparently related agents may reveal different patterns of biological activity.

The principal system used in the studies to be reported here was the pregnant rat and its fetuses. There are several reports on the normal development of the rat fetus,[1-4] and many drugs and modified diets have been shown to produce developmental abnormalities.[5-8] Furthermore, nitrogen mustard (HN2) will produce consistent teratogenic effects on the fetal rat,[9, 10] a system that thus suggested itself for the study of the comparative activity of other polyfunctional alkylating agents.

Three important variables involved in this system are: the fetus, the mother, and the pharmacological properties of the test drug. While these variables cannot be evaluated adequately from the limited data available, they must be considered in the interpretation of our results. The developing fetus presents a complex and evolving organism, and brief exposure to a noxious agent may produce a transient and specific action on active processes that subsequently may be magnified into extensive and specific developmental abnormalities in fetuses surviving to the end of gestation. While a specific teratogenic effect is a function, in part, of the stage of development when a noxious agent is applied, the effect is also related to the type of agent used.

Another factor is the close relationship between the mother and fetus. This involves a consideration of the toxic effects of the drug on the mother; its metabolism, detoxification, and excretion; the possible presence of protective substances in the mother; its effects on her endocrine system; and the efficiency with which the drug or its metabolites are transmitted to the fetus. Furthermore, drugs with the same type of biochemical action may, because of prosthetic groups of unique chemical configuration, have different properties, such as solubilities, metabolic fates, or rates of transport into cells, that may influence their pattern of biological effects.

Preliminary results from the comparison of several polyfunctional alkylating agents were available also on two systems in the chick embryo.[11] In one system the drugs were tested for teratogenic activity by injection into

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the yolk sac of the 4-day embryo,[12] in which it had previously been shown that HN2 had a mild and inconstant teratogenic effect.[13] In the other, the chick embryo bearing the transplantable mouse sarcoma 180 on the chorioallantoic membrane was injected with the test agent by the yolk-sac route on the twelfth day of incubation; in this system HN2 is lethal to the tumor and produces striking cytological changes in the tumor cells at doses that are relatively nontoxic to the embryo.[14, 15]

Materials and Methods

Pregnant rats. Previously unmated rats, Wistar strain, were placed in cages with males for 12 hours. Those found to have vaginal plugs were isolated and regarded as zero days pregnant. This method yielded 55 per cent true pregnancies, as determined by the percentage of these rats coming to term with viable or resorbing fetuses. On the twelfth day of gestation the presumably pregnant rats were divided into groups and treated with various dose levels of the test drugs or X rays. The drugs were given as a single intraperitoneal dose on a basis of mg./kg. of body weight; controls received the diluent.

The rats were sacrificed on the twenty-first day of gestation, 9 days after treatment. The obviously nonfertile rats were discarded. In the pregnant rats, the implantation sites were counted and the living fetuses were grossly described, weighed, and measured. They were then cleared, and the osseous skeleton was stained with alizarin red S. The mother was examined and, in some cases, the spleen and thymus were weighed and the femoral bone marrow was removed and fixed for microscopic examination.

Chick embryo. Fertile White Leghorn eggs were obtained from a commercial source and incubated at 38° C. The test chemicals, at various dose levels, were injected into the yolk sac of 4-day-old embryos. Those surviving to 18 days of incubation were sacrificed and weighed; the embryos were cleared, and the osseous skeleton stained with alizarin red S.

Sarcoma 180. Single fragments of mouse sarcoma 180 from tumor-bearing mice were explanted to the chorioallantoic membrane of the 8-day egg. On the twelfth day, groups of eggs with apparently viable tumors were selected and injected by the yolk-sac route with the test chemicals. On the eighteenth day of incubation the eggs were sacrificed, the embryos examined for gross abnormalities, and the spleens weighed. The tumors were excised from the chorioallantoic membrane, weighed, measured, and fixed in formalin for microscopic study, and a fragment was assayed in mice for viability.

Agents used. The agents used in this study are shown in FIGURE 1, with their chemical structures.* HN2, TEM, and Thio-TEPA were dissolved in physiological saline; chlorambucil and Myleran were suspended in arachis oil for the rats and in 0.5 per cent solution of carboxymethylcellulose (CMC) for the eggs. The X-ray factors used in treating the pregnant rats were 180 KVP X rays, 30 cm. target distance, HVL 0.8 mm. Cu, and the dose rate varied on different runs from 55 to 138 r per minute. All doses are in air at the position of the rat.

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* We acknowledge with appreciation the following sources of compounds: Mustargen (HN2), Merck Sharp & Dohme, Division of Merck and Co., Inc., Philadelphia, Pa.; TEM and Thio-TEPA, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.; chlorambucil, Boots Pure Drug Co., Ltd., Nottingham, England; and Myleran, Wellcome Research Laboratories, Tuckahoe, N. Y.

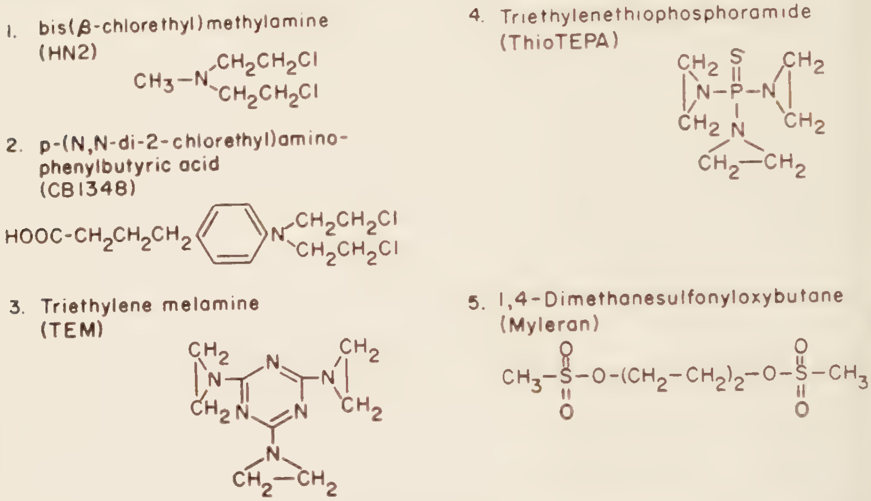
1. bis(β-chlorethyl)methylamine
(HN2)
2. p-(N,N-di-2-chlorethyl)aminophenylbutyric acid
(CB1348)
3. Triethylene melamine
(TEM)
4. Triethylenethiophosphoramide
(ThioTEPA)
5. 1,4-Dimethanesulfonyloxybutane
(Myleran)

FIGURE 1. Chemical and structural formulas of the alkylating agents used in this study.

Results

EFFECTS OF THE ALKYLATING AGENTS AND X RAYS ON THE PREGNANT RAT AND ON FETAL DEVELOPMENT

The results of these experiments, with detailed legends, are presented in FIGURES 2 to 6 and TABLES 1 and 2.

Toxicological Data

Dosage levels producing various effects on the mother and fetus are summarized in FIGURE 2. The dosage reference point is the litter LD_{50} at 12 days of gestation, or the dose that, given intraperitoneally to the mother, can be expected to destroy the majority of fetuses in 50 per cent of the treated litters. The estimated litter LD_{50}'s at 12 days and the number of pregnant rats treated in this dose range were as follows:

	Estimated litter LD_{50}	Number pregnant rats
HN2	0.7 mg./kg.	16
TEM	0.55 mg./kg.	21
Thio-TEPA	5.0 mg./kg.	26
Chlorambucil	10.0 mg./kg.	20
Myleran	34.0 mg./kg.	18
X rays	250 r	13

While HN2 is more toxic to the fetus earlier in development, the twelfth day of gestation was selected because HN2 given at this time produces its

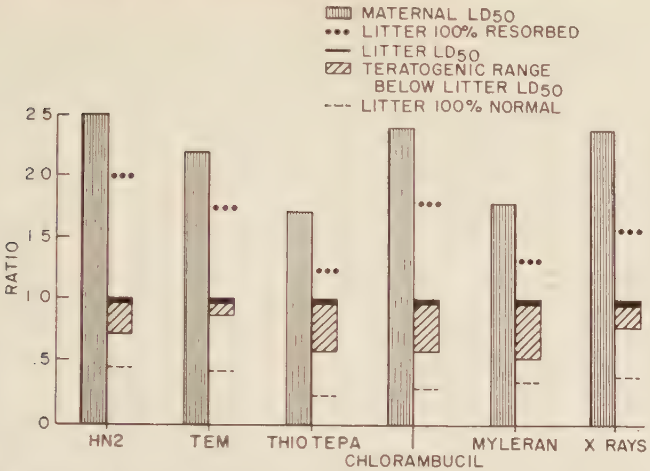

FIGURE 2. Ratio of dosage of alkylating agents and X rays given pregnant rats on the twelfth day of gestation to produce various effects on mother and fetus to estimated litter LD$_{50}$.

Litter LD$_{50}$. A *positive litter effect* is defined as a dose that is lethal to a majority of the fetuses by the twenty-first day of gestation. At this time half or more of the implantation sites would contain dead or resorbing fetuses. The *litter LD$_{50}$* is the dose calculated to produce a *positive litter effect* in 50 per cent of the treated mother rats. This value is taken as 1.0 for each compound, and the doses producing other effects are recorded as ratios of the *litter LD$_{50}$*.

Maternal LD$_{50}$. These were estimated for each compound from the toxicity data reported in the literature on nonpregnant rats, and were confirmed in our laboratory on small groups of pregnant rats. Pregnancy did not seem to affect the toxicity of the alkylating agents. In determining the maternal LD$_{50}$ for a drug given at the twelfth day of gestation, the observation period can be only 10 days, since the rats were sacrificed or littered at this time. On the basis of the 10 day observation period, the estimated maternal LD$_{50}$ ranged from 2.5 times the litter LD$_{50}$ for HN2, chlorambucil, and X rays to 2.25 for TEM and 1.75 times the litter LD$_{50}$ for Thio TEPA and Myleran. Myleran characteristically causes delayed deaths 14 to 17 days after injection in nonpregnant rats. Therefore, the dose of Myleran lethal to 50 per cent of pregnant rats within 10 days is larger than the LD$_{50}$ reported in the literature.

Litter 100 per cent resorbed. The dose giving 100 per cent litter resorptions was from 1.25 to 2 times the litter LD$_{50}$ for the drugs tested, but always less than the maternal LD$_{50}$.

The teratogenic range below litter LD$_{50}$. This is charted as the dose range between litter LD$_{50}$ and the lowest dose given in this study at which teratogenic effects were observed.

Litter 100 per cent normal. This is the highest dose administered in these studies that failed to produce teratogenic effects by our methods of observation. The litter 100 per cent normal is about one third to one half the litter LD$_{50}$ for the alkylating agents and X rays.

most consistent gross teratogenic effects. The ratios among the twelfth-day litter LD$_{50}$, the dose causing 100 per cent litter resorption, the minimum dose producing teratogenic effects (teratogenic dose range), and the maternal LD$_{50}$ are presented in FIGURE 2.

The relationship between the maternal and litter LD$_{50}$'s at 12 days for the polyfunctional alkylating agents and X rays is within the fairly narrow range of 2.5 to 1.75:1. It is therefore of interest to contrast this result with data obtained on several antimetabolites that also caused lethal and/or teratogenic effects on the rat fetus when given on the twelfth day (FIGURE 3).[8] Thiadiazole (2-ethylamino-1,3,4-thiadiazole) and 6-aminonicotinamide both show a ratio of litter to maternal LD_{50} in the range of 1:1 when administered on the twelfth day. DON (6-diazo-5-oxy-L-norleucine), azaserine (O-diazoacetyl-L-serine), and Aminopterin (4-aminopteroylglutamic acid) were far more toxic to the fetus, with the ratios of litter to maternal LD_{50}'s of 530:1, 21:1, and 6 to 8:1, respectively. Teratogenic effects were seen regularly

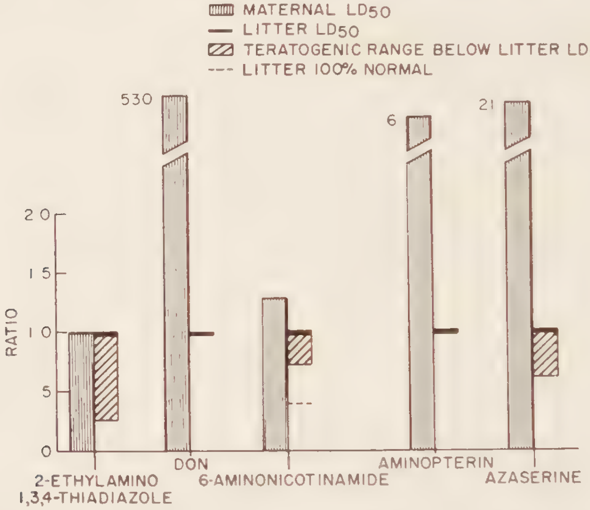

FIGURE 3. Ratio of dosage of several antimetabolites injected intraperitoneally into pregnant rats on the twelfth day of gestation to produce various effects on mother and fetus to estimated litter LD_{50} (see FIGURE 2 for an explanation of the symbols and effects described). Brief summaries of pharmacological data on these agents, which have been reported in more detail elsewhere,[8] are as follows:

2-Ethylamino-1,3,4-thiadiazole is a water-soluble chemical. It inhibits the growth of transplantable tumors, and this can be prevented by the simultaneous administration of nicotinamide. In pregnant rats a single injection administered on the twelfth day caused gross and skeletal abnormalities of the fetus at doses between 0.25 to 1.0 times the maternal LD_{50} dose (50 to 200 mg./kg.). Some of the abnormalities at 100 mg./kg. included cleft palate, short lower jaws, short tails, and syndactyly of the front and rear feet. Some of the pregnant animals surviving the maternal LD_{50} contained viable fetuses, indicating that the litter LD_{50} is in the same range as the maternal LD_{50}.

DON (*6-diazo-5-oxy-L-norleucine*) is a water-soluble chemical. It is inhibitory to a wide spectrum of transplantable rat and mouse tumors, and it produces developmental abnormalities in the chick embryo. Some of its effects are prevented by the administration of adenine and 4-amino-5-imidazole carboxamide. In rats the maternal LD_{50} of DON is 80 mg./kg. When 0.2 mg./kg. was given on the twelfth day the fetuses were all resorbed, but 0.1 mg./kg. seemed to have no adverse effect on fetal development and survival. The ratio of litter to maternal LD_{50} for DON is 530:1.

6-Aminonicotinamide is a powerful antagonist of nicotinamide. It is poorly soluble in water and prepared as a suspension in 0.5 per cent CMC. The maternal LD_{50} dose for the rat is 8 to 15 mg./kg., being somewhat lower earlier in gestation. At LD_{50} doses, toxic manifestations in the adult include conjunctival hemorrhages (bloody eyes), paralysis of the rear extremities, and weight loss (the paralysis persisted during the life of one animal with the thiadiazole, 6-aminonicotinamide, and azaserine, but were rarely seen with DON and Aminopterin. The latter group either caused fetal resorption, or the surviving fetuses appeared to be normal. Some of the teratogenic effects of the antimetabolites differed qualitatively from those produced by the polyfunctional alkylating agents (FIGURE 3).

In the range of the litter LD_{50}, evidence of toxicity was seen in the mother rat, and the following criteria of maternal toxicity were used:

Weight gain. From the twelfth to twenty-first days of gestation the control pregnant rats each gained an average of 80 gm. The mothers treated with the alkylating agents at doses of less than one half the litter LD_{50} showed normal weight gain. In the range of 0.75 to 1.25 times the litter LD_{50}, weight gain did not ordinarily occur. This may be explained by the high incidence of fetal resorptions as well as by maternal intoxication. Above 1.5 times the litter LD_{50}, the alkylating compounds all caused weight loss. At doses above 2.0 times the litter LD_{50} the weight gain was severe. X rays, as compared to the alkylating agents, caused interference with maternal weight gain at smaller fractions of the litter LD_{50}.

Spleen weight. HN2, Thio-TEPA, chlorambucil, and TEM had no appreciable effect on the maternal spleen weight in the range of the litter LD_{50}. At 1.5 times the litter LD_{50} a decrease in spleen weight was noted with Thio-TEPA, chlorambucil, and HN2, but not with TEM. Both Myleran and X rays, in the range of the litter LD_{50} or higher, caused an appreciable decrease in spleen weight.

Thymus weight. HN2, Thio-TEPA, chlorambucil, TEM, and X rays had no striking effect on thymus weight in the range of the litter LD_{50}. At 2.0 times the litter LD_{50} all agents caused a definite decrease in thymus weight. Myleran diminished thymus weight at doses below the litter LD_{50}.

Bone marrow depletion. The femoral bone marrow, obtained at the end of gestation, was examined microscopically for conspicuous evidence of depletion of the hematopoietic elements. HN2 treatment with all doses at 12 days resulted in only slight depression of marrow at 21 days. Myleran

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that survived for 3 months and was then sacrificed). These effects can be prevented by the simultaneous administration of a 100-mg./kg. dose of nicotinamide, but administration of the same dose of nicotinamide after paralysis had developed did not correct it. Eight-mg./kg. doses of 6-aminonicotinamide to the pregnant female on the twelfth day produced maternal weight loss and inhibition of fetal weight, syndactyly, harelips, and cleft palates. Doses of 5 mg./kg. produced no fetal resorptions or abnormalities. The ratio of litter to maternal LD_{50} is almost 1:1.

Aminopterin (4-aminopteroylglutamic acid) is a water soluble antagonist of folic acid. The LD_{50} for the pregnant rat is approximately 3 mg./kg. The litter LD_{50} on the twelfth day of gestation is about 0.4 mg./kg. It is interesting to note that fetuses that survive are usually normal, a situation similar to that seen with DON. The ratio of litter to maternal LD_{50} is from 6 to 8:1.

Azaserine (O-diazo-acetyl-L-serine) is a water-soluble chemical, closely related chemically and in its pharmacological effects to DON, but less active by weight. The maternal LD_{50} is 75 to 100 mg./kg. as a single dose. The litter LD_{50} was 3.0 mg./kg. on the twelfth day, and surviving fetuses usually showed gross and skeletal abnormalities. The latter consisted of fusion of vertebral bodies, small pelves, and specific deletion of femurs and fibulas. The ratio of fetal to maternal LD_{50} for azaserine is 21:1.

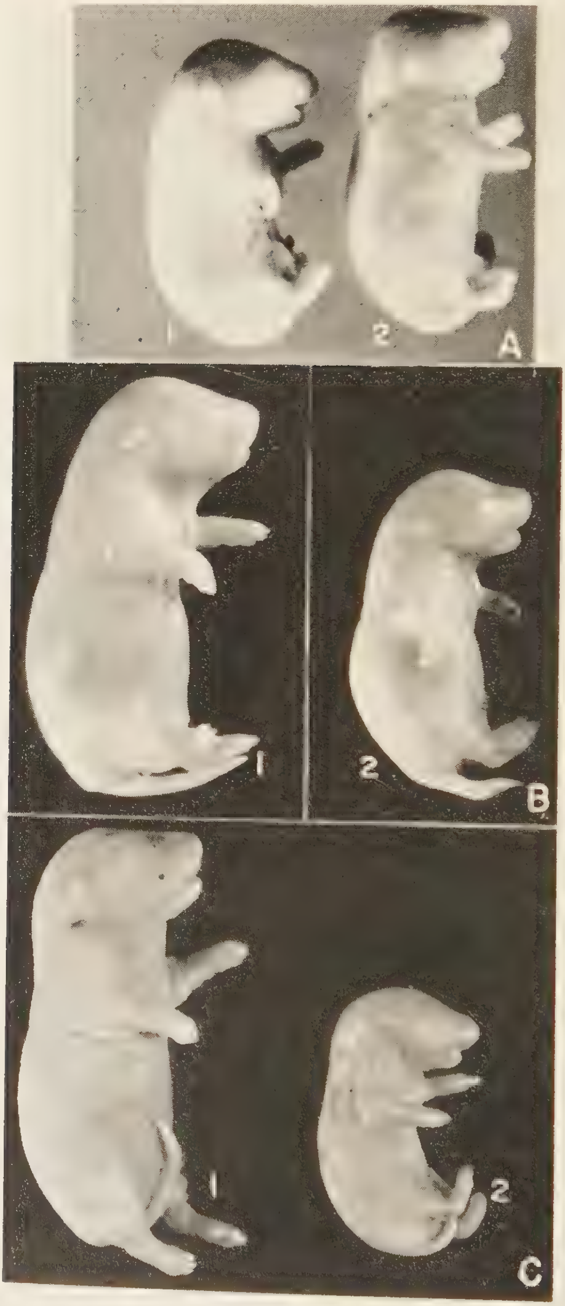

Figure 4

caused severe marrow depletion at one half the litter LD_{50}; Thio-TEPA, chlorambucil, and X rays at 1.5 times; and TEM at 2.0 times the litter LD_{50}.*

Myleran, in relation to the litter LD_{50}, produced more conspicuous decrease in spleen and thymus weight and in bone marrow cellularity than the other alkylating agents. Since the delayed deaths produced by Myleran occur considerably later than with the other alkylating drugs, an LD_{50} figure for Myleran, determined on the basis of a 9-day observation period, is unduly high. If the maternal LD_{50} figure for Myleran were based on a 20-day observation period, the effects of this dose on the mother would probably be quantitatively more consistent with effects produced by maternal LD_{50} doses of the other alkylating agents.

Gross Developmental Abnormalities

Characteristic abnormalities observed in fetuses surviving to term after exposure to the alkylating agents or X rays on the twelfth day of gestation are shown in FIGURES 4 and 5. The gross abnormalities observed are listed in order of their frequency in TABLE 1. These observations were made on fetuses that survived in the range of the litter LD_{50}. Because the more severe defects were found in the surviving members of litters in which the majority had been resorbed, a distinction is made in TABLE 1 between fetuses examined from litters in which the majority survived ($<LD_{50}$) and in which the majority were resorbed ($>LD_{50}$). Defects of the digits and tails were most common, and these occurred in fetuses otherwise showing only slight retardation of weight, as well as those with severe stunting and extensive abnormalities. Encephaloceles of varying degrees of severity were seen with all agents except Myleran; and cleft palates, a common occurrence with other agents, were conspicuously absent in fetuses treated with TEM and Thio-TEPA, even at doses producing weight inhibition and encephalocoeles. Massive edema was most severe in fetuses receiving X rays. Among the chemicals used, fetal edema occurred most frequently with chlorambucil.

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* We express our appreciation to Gisela Stecher, who examined the bone marrow sections.

FIGURE 4. Photographs of freshly sacrificed 21-day fetuses. They include control specimens and selected examples of fetuses that survived 9 days after the mothers were treated with HN2, Thio TEPA, and Myleran, respectively, on the twelfth day of gestation.

The *A* fetuses were treated with a 0.7-mg./kg. dosage of HN2. No control fetus is shown. The litter contained 11 fetuses; all survived to 21 days. Weight and length decreased; the fetuses are slightly edematous and show minimal encephaloceles. The upper and lower jaws are short, and protruding tongues can be seen. Cleft palates are present, but are not demonstrated in this photograph. In *A1* the right forelimb is curved. Both fetuses show short tails and syndactyly of the front and rear digits.

The *B* fetuses received a 4.0-mg. kg. dosage of Thio TEPA. *B1* is the control. Eight fetuses of a litter of 9 survived to 21 days. Weight and length decreased. An encephalocele is present, but not visible in this photograph. The lower jaw is short. The right forelimb is rotated backward. Syndactyly is evident, and the tail is short and bent.

The *C* fetuses received a 34.0-mg./kg. dosage of Myleran. *C1* is the control. The litter contained 11 fetuses; all survived to 21 days. Weight and length markedly decreased, and a short lower jaw was observed. Cleft palate was present, but not visible in the photograph. The digits of the forelimbs are fused to give a pointed paw, a deformity characteristic for Myleran. The rear limbs show a slight degree of syndactyly.

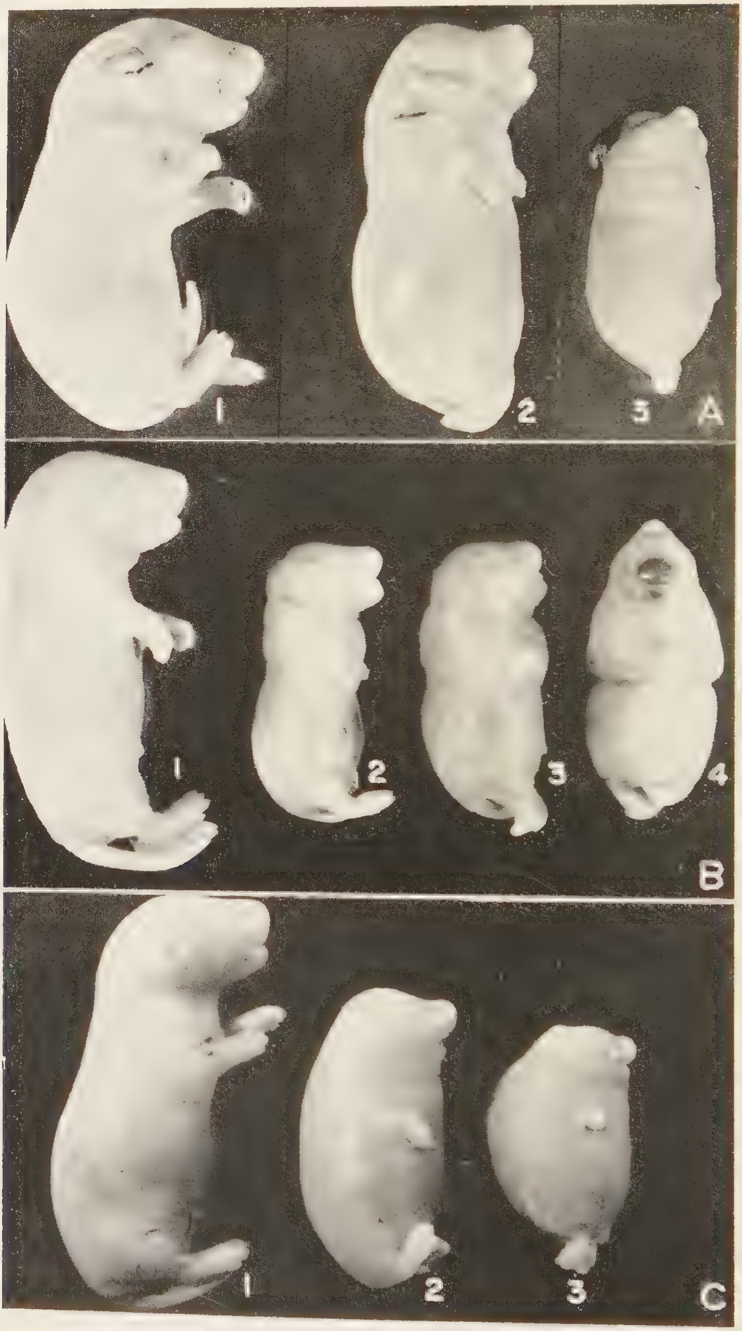

FIGURE 5

Abnormalities of the Osseous System

FIGURE 6 shows examples of skeletal abnormalities seen with the alkylating agents and X rays. Consistent abnormalities were observed in the ribs and vertebrae (FIGURE 6A), the limbs (FIGURE 6B), and the skull and sternebrae (FIGURE 6C). The fetuses were usually retarded, and bone stains often failed to show ossification of the digits. The characteristic deformities of the long bones and ribs could be graded according to three degrees of severity. The mildest effect was a curve or bend of the bone; the intermediate effect was ossification of small portions of bone; the most severe effect was absence of the bone.

In TABLE 2 the skeletal abnormalities, in order of frequency of occurrence, are listed for each agent. The skeletons included in the analysis in TABLE 2 are from litters with at least one bone abnormality. With few exceptions, all agents produced similar types of defects. Myleran produced no circular occipital defects (also, encephaloceles were not found grossly). Instead, some fetuses revealed an abnormal or absent supraoccipital bone. The most consistent teratogenic effects were produced by X rays and chlorambucil.

TERATOGENIC EFFECTS OF THE POLYFUNCTIONAL ALKYLATING AGENTS ON THE 4-DAY CHICK EMBRYO

TABLE 3 shows the approximate LD_{50} dosage of the alkylating drugs when injected into the yolk sac of the 4-day embryo, and the degree of teratogenic

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FIGURE 5. Photographs of freshly sacrificed 21-day fetuses. They include control specimens and selected examples of fetuses that survived 9 days after the mothers were treated with chlorambucil, TEM, and X rays, respectively, on the twelfth day of gestation.

The *A* fetuses were treated with chlorambucil. *A1* is the control. *A2* received a dosage of 6 mg./kg. All 14 fetuses of this litter survived to 21 days. Weight and length are slightly decreased. An abnormally shaped skull with upturned nose, small ears, short forelimbs, clubbed rear limbs, fused digits, and short tail are evident. *A3* received a 12-mg./kg. dosage. Four fetuses from a litter of 12 survived to 21 days. Weight and length are decreased. Edema is present. The brain protrudes through the occipital area of the skull, which is reduced in size. Ears, extremities, and tail are rudimentary.

The *B* fetuses received a 0.55-mg./kg. dosage of TEM. *B1* is the control. The three fetuses shown here are the only ones that survived to 21 days from a litter of 9. Weight and length are decreased. The fetuses are edematous. *B2* is a lateral view demonstrating the presence of an encephalocoele and protruding tongue. The forelimbs are flipperlike, the rear limbs are curved, and the tail is short. *B3* is more severely affected and demonstrates, in addition, curving of the rear limbs and syndactyly. *B4* is a dorsal view, and demonstrates the encephalocele of an equally affected littermate.

The *C* fetuses received X rays. *C1* is the control. *C2* received a dosage of 200 r, and is from a litter of 12, of which all survived to 21 days. Weight and length decreased. Edema, shortened upper and lower jaws, cleft palate (not shown in this photograph), small ears, and shortened limbs and tail are evident. The fused digits in the front paws form a rectangle. *C3* received a dosage of 300 r. From a litter of 11, 4 fetuses survived to 21 days. Weight and length are decreased. There is edema, an encephalocele and shortened upper and lower jaws, and small ears. A cleft palate is present, but cannot be seen in the photograph. The small forelimbs are obscured by the edematous body. Syndactylous digits form a bud. The hind limbs are loose, and the osseous skeleton is not found in stained preparations. The tail is shortened.

TABLE 1

INCIDENCE OF SELECTED GROSS ABNORMALITIES OF THE RAT FETUS PRODUCED BY INJECTING VARIOUS ALKYLATING AGENTS INTRAPERITONEALLY INTO THE MOTHER ON THE TWELFTH DAY OF GESTATION

	HN2		TEM		Thio-TEPA		Chlorambucil		Myleran		X ray	
	$<LD_{50}$*	$>LD_{50}$†	$<LD_{50}$	$>LD_{50}$	$<LD_{50}$	$>LD_{50}$	$<LD_{50}$	$>LD_{50}$	$<LD_{50}$	$>LD_{50}$	$<LD_{50}$	$>LD_{50}$
Number of fetuses examined	66	61	25	35	73	16	59	47	105	17	39	14
Total‡	127		60		89		106		122		53	
Number with specific abnormalities												
Decreased weight and length	66	61	25	35	36	16	59	47	105	17	39	14
Syndactylous forepaws	11	7	24	23	29	13	44	35	88	17	37	14
Short kinky tail	0	6	2	8	3	5	54	38	88	16	30	14
Encephalocele	0	3	6	5	1	3	22	44	0	0	4	14
Syndactylous rear paws	3	2	2	7	0	1	17	38	81	17	34	14
Cleft palate	4	2	0	0	0	0	10	38	12	0	8	14
Edema 4+	0	2	3	9	0	2	7	33	0	1	14	14

* $<LD_{50}$: mothers received a dose below the litter LD_{50}, but showing teratogenic activity.

† $>LD_{50}$: mothers received a dose at or above the litter LD_{50}.

‡ Total number of fetuses examined. These fetuses were obtained from litters whose mothers received a dose of a drug that had been demonstrated to produce consistent incidence of teratogenic activity.

activity. The LD_{50} is calculated on the basis of 10-day survival. The embryos alive at 18 days were sacrificed, weighed, and examined for gross abnormalities. Chlorambucil was the most consistent and active as a teratogenic agent in the group. An affected 18-day embryo is shown in FIGURE 7. Myleran and chlorambucil produced weight inhibition in the embryos observed at 18 days.

Effects of the Polyfunctional Alkylating Agents on the Sarcoma 180 Growing on the Chorioallantoic Membrane of the Chick Embryo

The effects of the various alkylating agents injected at two fifths of their LD_{50} dose by the yolk-sac route in the 12-day embryo are summarized in TABLE 4. The embryos were sacrificed at 18 days. At this dose there was less than 10 per cent decrease in body weight. The embryos treated with HN2, chlorambucil, and TEM occasionally showed inhibition of feather growth; spleen weight was decreased by TEM, Thio-TEPA, and Myleran.

At two fifths of the embryo LD_{50} dose, all agents produced severe damage to the sarcoma 180, in that its growth was inhibited in the egg, it failed to

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Description of Gross Abnormalities Enumerated in Table 1

(1) *Decreased weight and length.* The abnormal fetuses were usually smaller than controls although, if only a single fetus survived in a litter, it tended to be normal in size.

(2) *Syndactylous front paws* (FIGURE 4, B2 AND C2). Abnormalities of the digits of the forepaws appeared to be the commonest abnormality. They occurred among all the fetuses treated with the alkylating agents. There were various degrees of syndactyly. Occasionally one of the digits was hemorrhagic or one was missing. The Myleran-treated animals (FIGURE 4, C2) usually showed a pointed paw, with retardation of the first and second and the fourth and fifth digits making the third appear long. In the fetuses showing syndactyly, the cleared specimens did not show ossification of the metacarpals and phalanges. Cartilage stains of the paws would have been revealing here, but they were not done.

(3) *Short kinky tail* (FIGURE 4, B2; FIGURE 5, B4). The tails were occasionally absent, shortened with or without a sharp bend, or normal. The calcification of caudal vertebrae in cleared specimens was delayed.

(4) *Encephalocele* (FIGURE 5, A3, B2, AND B4). A defect of the occiput was seen in fetuses treated with all agents except Myleran. The occurrence of encephaloceles was usually associated with the higher doses. At lower doses, or in less severely affected fetuses at higher doses, there was a pin-point size blister or an area of ecchymosis. In cleared specimens the defect of the bones often occurred without any detectable gross abnormality of the head (TABLE 2). More severely affected fetuses had protruding brain tissue of 3 to 5 mm. in diameter. The largest encephaloceles were seen with chlorambucil (FIGURE 5, A3).

(5) *Syndactylous rear paws.* The rear paws were affected much the same as the front paws, but less frequently, with the exception of fetuses treated with chlorambucil and Myleran.

(6) *Cleft palate.* Cleft palate was seen in fetuses whose mothers were treated with HN2, chlorambucil, Myleran, and X rays, but not with Thio-TEPA or TEM, even in fetuses surviving doses of the litter LD_{50}.

(7) *Edema* (FIGURE 5, C3). Edema was present in slight degree in many fetuses, but it was recorded only if it was severe. Edema was most conspicuous after X rays, and least after Myleran.

TABLE 2

INCIDENCE OF SELECTED SKELETAL ABNORMALITIES OF THE RAT FETUS PRODUCED BY INJECTING VARIOUS ALKYLATING AGENTS INTRAPERITONEALLY INTO THE MOTHER ON THE TWELFTH DAY OF GESTATION

	HN2		TEM		Thio-TEPA		Chlorambucil		Myleran		X ray	
	$<LD_{50}$*	$>LD_{50}$†	$<LD_{50}$	$>LD_{50}$	$<LD_{50}$	$>LD_{50}$	$<LD_{50}$	$>LD_{50}$	$<LD_{50}$	$>LD_{50}$	$<LD_{50}$	$>LD_{50}$
Number of fetuses examined	51	57	25	29	20	14	52	30	66	16	37	7
Total‡	108		54		34		82		82		44	
	Number with specific abnormalities											
Ribs	18	33	13	3	10	2	28	30	50	16	37	7
Sternum	25	14	8	2	13	2	24	28	35	16	19	7
Zygoma	5	9	11	2	9	2	19	26	19	0	37	7
Occiput	13	16	20	3	8	2	17	30	0	0	23	7
Ulna	6	9	10	4	0	1	20	30	3	11	37	7
Scapula	10	16	4	2	1	0	11	26	30	15	8	7
Radius	3	7	7	2	0	0	11	26	3	12	37	7
Fibula	4	1	8	3	0	0	15	29	11	11	37	7
Tibia	0	0	6	2	1	0	11	23	0	0	37	7
Humerus	0	6	0	0	0	0	7	16	10	8	37	7
Femur	2	0	1	0	0	0	1	6	0	0	37	7

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* $<LD_{50}$: mothers received a dose below the litter LD_{50}, but showing teratogenic activity.

† $>LD_{50}$: mothers received a dose at or above the litter LD_{50}.

‡ Total number of fetuses examined. These fetuses were obtained from litters whose mothers received a dose of a drug that had been demonstrated to produce consistent incidence of teratogenic activity.

grow when bioassayed into mice, and characteristic signs of cytologic injury occurred in the treated tumors (FIGURE 8).

Discussion

The polyfunctional alkylating agents have produced developmental abnormalities in *Drosophila* exposed to HN2 during the larval stage,[16,17] in *Amblystoma* larvae,[18] and in mouse [19,20] and rat fetuses.[9,10,21] The most striking teratogenic effects caused by these agents in mice and rats occurred when the mother was treated on the twelfth to thirteenth days of gestation; presumably at this period differentiation is largely complete and organogenesis is in progress.

It is presumed that these agents act directly on the developing embryo. This concept is supported by the fact that they are teratogenic *in vitro*, and

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DESCRIPTION OF SKELETAL ABNORMALITIES ENUMERATED IN TABLE 2

(1) *Ribs.* The skeletal abnormality seen most often involved the ribs, usually from the sixth caudally, but also in the first half, as well. With lower doses, or in less severely affected animals at higher doses, the least severe form was a sharp angulation at the junction of the posterior and middle thirds of the rib. In more severe forms the rib was interrupted to form a dotted appearance, or some or all of the ribs were absent entirely. Some animals showed a combination of all of these defects in different ribs (FIGURE 6A).

(2) *Sternum.* The sternebrae were usually retarded in development, and the fifth sternebra was usually missing. Most severe defects consisted of premature fusion of the sternebrae into a continuous plate, or the individual centers were double and one-half displaced forward or backward.

(3 and 4) *Zygoma and Occiput* (FIGURE 6C2). In the area of the encephaloceles, observed grossly, the skulls showed a circular defect involving parietal, interparietal, supraoccipital, and occipital bones. In many severely retarded fetuses, in addition to the skull defects, the bones were small and poorly calcified. Although Myleran produced no encephaloceles or circular occipital defects, some animals, at high doses, had an abnormally shaped, defective, or absent supraoccipital bone. This was not seen with the other alkylating agents. A second bony defect noted with all the agents was a failure of complete calcification of the zygoma. This involved the squamous as well as maxillary portions.

(5) *Ulna* (FIGURE 6, B2 AND B3). The ulna was the most commonly affected long bone. The usual defect appeared as a curved bone; more severe defects were deletion of a portion of the bone, or two spots of calcification (this was also seen in the ribs), or complete absence of the ulna. The latter, of course, appeared in the more severely affected animals. The ulnar defect was associated with the abnormal position of the front extremity described on gross examination. Thio-TEPA produced the fewest long-bone defects.

(6) *Scapula.* The scapula was affected by all the agents, but least frequently by Thio-TEPA. The scapular defect was sometimes confined to the spine, which was abnormally long or short. The commonest defect was a fairly sharp angulation toward the convex surface of the scapula at about the midpoint of the longest axis.

(7) *Radius.* The radius was also affected by all the agents except Thio-TEPA. The most frequently observed abnormality was a curvature toward the volar surface. In a few cases the radius was absent.

(8) *Fibula.* The fibula was the most frequently affected long bone of the rear extremity. It was decreased in size or missing. Curvature was not seen.

(9) *Tibia.* The tibia was less frequently affected; it was fused to the femur, short, or absent. Defects of the tibia were seen principally with X rays, chlorambucil, and TEM.

(10 and 11) *Humerus and femur.* The humerus and femur were the least affected bones by all agents except X rays. The abnormalities were a decrease in size, absence or, in some cases, a fusion at right angles to the radius or humerus, respectively. The latter was seen principally with X rays.

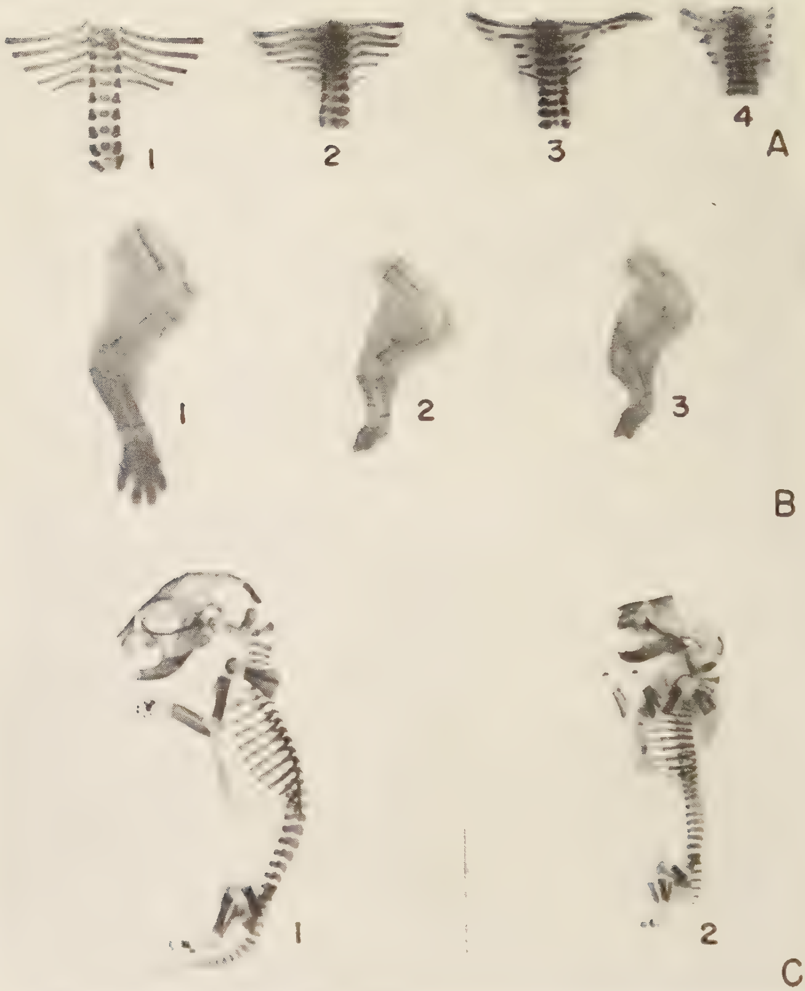

Figure 6. Alizarin-stained skeletal preparations of 21-day fetuses from pregnant rats treated with Thio-TEPA, TEM, and HN2 on the twelfth day of gestation.

(A) *Lower ribs and attached thoracic and lumbar vertebrae* (down to the third to fifth). The agent was Thio-TEPA, 4.0 mg./kg. *A1* is the control. In *A2* the mildest rib deformity is an angulation of the ribs at the junction of the posterior and middle thirds. *A3* shows a more severely affected member of this litter, showing interruption of ossification of the ribs. *A4* is the severest deformity, showing some dotted calcifications for ribs and the actual absence of some ribs.

(B) *Forelimb.* The agent was TEM, 0.54 mg./kg. *B1* is the control. In *B2* the limb is short and the bones are smaller. The radius is curved; the ulna is proportionately smaller than the radius. While syndactyly of the soft tissues is evident in this specimen, none of the bones of the paw is calcified. *B3* is a more severely affected fetus from the same litter. The radius is small and curved, and the ulna is missing. Again, none of the bones is calcified in the paw.

(C) *Whole skeleton.* The agent was HN2, 0.7 mg./kg. *C1* is the control. *C2*, the lateral view, shows the circular defect of the bones at the crown of the head involving parietal, interparietal, occipital, and supraoccipital bones. In this specimen, before clearing, an encephalocoele protruded through this defect. The fifth sternebra is absent. Cleft palate was present, but not visible in this view.

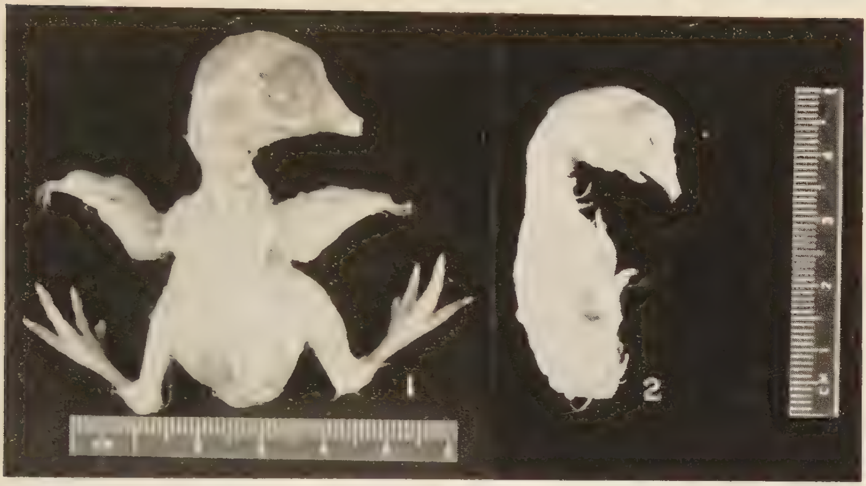

FIGURE 7. Chick embryos sacrificed at 18 days of incubation. Number 1 is the control; number 2 the chick injected into the yolk sac on the 4th day of incubation with 2.0 mg. per egg of chlorambucil. It shows stunting, shortened lower beak, marked retardation of the extremities, and missing toes.

TABLE 3
COMPARISON OF EFFECTS OF ALKYLATING AGENTS ON THE 4-DAY CHICK EMBRYO

Compound	LD_{50}/egg (mg./egg)	Weight inhibition	Teratogenic activity
HN2	0.01	−	±
Chlorambucil	1.0 to 2.0	+	++
TEM	0.002 to 0.005	−	−
Thio-TEPA	0.0075 to 0.01	−	−
Myleran	0.3	+	−

TABLE 4
COMPARISON OF EFFECTS OF ALKYLATING AGENTS AGAINST SARCOMA 180, GIVEN AT TWO FIFTHS OF THE ESTIMATED LD_{50} FOR 12-DAY EMBRYO

Compound	Estimated $2/5$ of LD_{50} mg./egg	Tumor wt.-mg. average	Histology	Embryo wt.-gm. average	Abnormal embryos	Spleen wt.-mg. average
Control		175	Good tumor	15.8	0/12	17.0
HN2	0.2	68	Damage 4+	14.6	3/6	14.0
Chlorambucil	0.6	43	Damage 4+	13.9	3/9	11.0
TEM	0.02	38	Damage 4+	12.5	5/10	3.5
Thio-TEPA	0.06	34	Damage 4+	14.9	0/8	6.6
Myleran	2.0	92	Damage 4+	12.8	0/8	4.5

that a direct placental or endocrine effect, to explain fetal abnormalities and death from the alkylating agents, has been considered and excluded in the mouse by Didcock *et al.*[20] and in the rat by Thiersch.[21] It appears likely that these agents affect susceptible cells in different ways,[18] and that certain types of cells are maximally sensitive at specific stages of development. HN2 probably acts primarily on the cell nucleus to interfere with new cell formation. While cells undergoing frequent division are most susceptible, there is also evidence that the marked susceptibility of some cells is not related to their mitotic activity. A proper exploration of the variety of teratogenic effects produced by HN2 and other alkylating agents would include a study of their toxicity patterns and teratogenic effects when given at various stages of gestation, and the examination of fetuses at frequent periods after treatment in order to observe the defects occurring in those doomed to be resorbed before term. This formidable problem is being

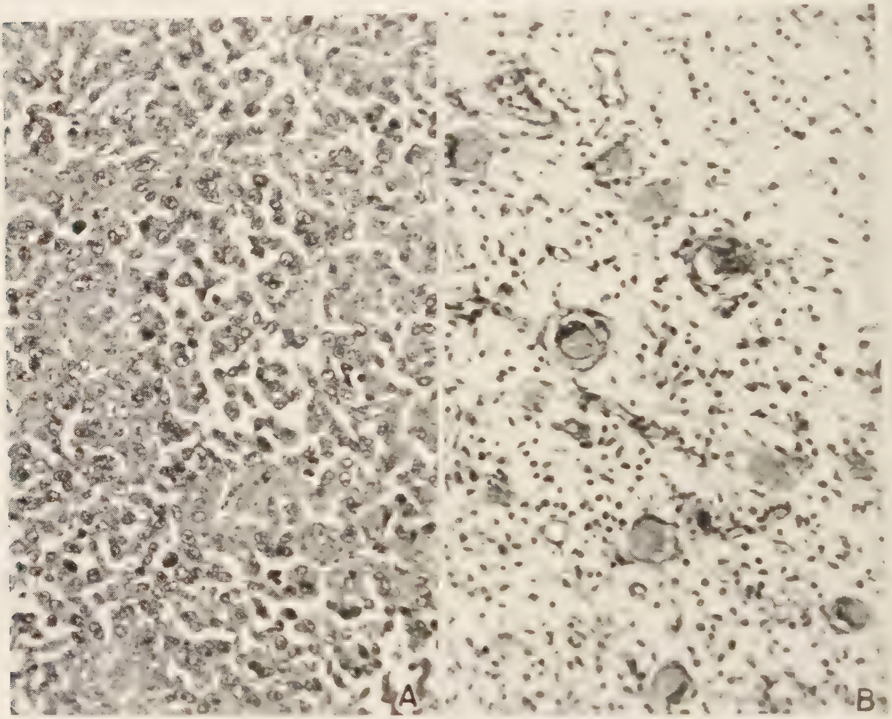

FIGURE 8. Sarcoma 180 (×200) grown on the chorioallantoic membrane of the chick embryo—implanted at 8 days of incubation, sacrificed at 18 days. (A) Tumor from control egg. (B) Tumor from egg treated with 0.6 mg. of chlorambucil on the twelfth day of incubation and sacrificed at the eighteenth day. Tumors from treated embryos weighed less than the controls (TABLE 4). The microscopic sections of treated tumors show a marked decrease in the number of cells. The treated tumor cells present are enlarged with abnormal nuclei, and there is an increase in interstitial connective tissue. Bioassays of the treated tumors in mice were negative.

investigated following irradiation; Hicks,[22] for example, has described changing patterns of defects in the rat fetus when it is irradiated at different stages of development.

In this report, however, we are concerned principally with the comparative effect of several alkylating agents on a specific system, the pregnant rat on the twelfth day of gestation. Do our data provide evidence that these agents have qualitatively different biological effects? Because they reported on a somewhat similar problem, we may quote from a study by Bodenstein and Goldin,[23] who used nine bifunctional analogues of HN2 at the same concentrations on *Amblystoma* larvae. These investigators conclude: "The observed differences in the amount of cellular destruction brought about by the different compounds are apparently reflections of differences in their effective potencies; hence the compounds differ, as far as their effects are concerned, only quantitatively but not qualitatively. This assumption seems to be well founded, especially in view of the fact that a series of nuclear responses similar to those provoked by the various kinds of mustards could also be brought about by appropriate concentrations of only one mustard compound."

The systems used in our study were not only more complicated but, in addition, they were exposed to more diversified forms of polyfunctional alkylating agents. Furthermore, we attempted to work at biologically equivalent doses of each agent. In the pregnant rat, all agents in the range of the maternal LD_{50} produced bone marrow depression and involution of lymphatic tissues. Furthermore, all agents showed a fairly uniform relationship between the maternal and litter LD_{50}. In relation to the litter LD_{50}, the teratogenic dose range was fairly similar for all compounds and, with a few exceptions, the resultant gross and skeletal abnormalities followed an impressively uniform pattern. Chlorambucil exhibited the most consistent teratogenic effects, whereas Myleran was least active. Possibly the low solubility of Myleran is an explanation for this, since it may penetrate the placenta slowly and in insufficient concentrations to produce acute changes. The greater teratogenic action of chlorambucil is more difficult to explain. It is noteworthy that the most severe and consistent teratogenic effects were produced by X rays; X rays penetrate all tissues fairly uniformly, and they do not present such problems as rate of absorption from the injection site, drug metabolism, placental transfer, and cellular penetration. In the case of the alkylating agents we can speculate only that such differences as those in chemical reactivity, solubility, distribution, blood levels, and metabolism influenced, in time and concentration, their availability to the responsive end cell, which is affected in a uniform manner.

This suggestion is supported by the experiments involving the sarcoma 180 explants on the chlorioallantoic membrane of the chick embryo. All of the polyfunctional alkylating agents studied produced a uniformly destructive effect on the tumor in terms of growth inhibition, loss of viability, and specific cytological damage. It is particularly important to note that these drugs destroy the sarcoma 180 at doses not lethal to the chick embryo. The selective toxicity of the alkylating agents to the sarcoma 180 (and this probably indicates a selective action on mammalian as compared to avian tissue) indicates that the susceptible cells respond in a uniform fashion when the alkylating agent reaches them in adequate concentrations.

Summary and Conclusions

(1) Five polyfunctional alkylating agents (HN2, chlorambucil, TEM, Thio-TEPA, and Myleran) were examined for their comparative effects on the pregnant rat and its fetuses, on the chick embryo, and on sarcoma 180 growing on the chorioallantoic membrane of the chick embryo.

(2) These agents have similar toxicological effects on the pregnant rat. In relation to the doses, given at the twelfth day of gestation, causing fetal resorption (measured as the litter LD_{50}), the maternal LD_{50}'s were 1.75 to 2.5 times larger. The surviving fetuses were examined at term for gross and skeletal abnormalities. The teratogenic effects that varied with dosage were fairly uniform for all compounds, although there were a few exceptions. Chlorambucil was the most consistently active teratogenic agent; Myleran was the least active.

(3) The group of alkylating agents, at doses of two fifths of the LD_{50} for the 12-day chick embryo, consistently inhibited the growth, damaged the cells (as observed cytologically), and destroyed the viability of the sarcoma 180 explanted to the chlorioallantoic membrane. At these doses the chick embryo was relatively unaffected.

(4) These results were compared with those from X rays. X rays were more consistently teratogenic in the rat fetus and chick embryo, but the gross and skeletal defects studied in the rat fetus were similar for X rays and the alkylating agents. The selective lethal effect of the alkylating agents for the sarcoma 180 growing in the fertile egg was not seen with X rays, which damaged the tumor severely only at doses causing lethal injury to the chick embryo.

References

1. Long, J. A. & P. L. Burlingame. 1938. The development of the external form of the rat, with some observations on the origin of the extraembryonic coelom and foetal membranes. Zoology. **43**: 143.
2. Nicholas, J. S. 1935. The normal stages in the development of the rat. Thesis. Osborn Zool. Lab. Yale Univ. New Haven, Conn.
3. Strong, R. M. 1926. The order, time and rate of ossification of the albino rat (*Mus norvegicus albinus*) skeleton. Am. J. Anat. **36**: 313.
4. Henneberg, B. 1937. Normentafeln zur Entwicklungsgeschichte der Wanderratte. *In* Normentafeln zur Entwicklungsgeschichten der Wirbeltiere. **15.** F. Keibel, Ed. Fischer. Jena, Germany.
5. Warkany J. 1954. Congenital malformations induced by maternal dietary deficiency; experiments and their interpretation. Harvey Lectures. Ser. **48**: 89.
6. Nelson, M. M. 1955. Mammalian fetal development and antimetabolites. *In* Antimetabolites and Cancer. C. P. Rhoads, Ed. Am. Assoc. Advance. Sci.
7. Wilson, J. G. 1954. Influence on the offspring of altered physiologic states during pregnancy in the rat. Ann. N. Y. Acad. Sci. **57**(5): 517.
8. Murphy, M. L., C. P. Dagg & D. A. Karnofsky. 1957. Comparison of teratogenic chemicals in the rat and chick embryos. Pediatrics. **19**: 701.

9. Haskin, D. 1948. Some effects of nitrogen mustard on the development of external body form in the fetal rat. Anat. Record. **493**: 511.
10. Murphy, M. L. & D. A. Karnofsky. 1956. Effect of azaserine and other growth-inhibiting agents on fetal development of the rat. Cancer. **9**: 955.
11. Karnofsky, D. A. & C. Lacon. Effects of various alkylating agents and X-rays on the growth and metastases from a transplantable mouse tumor (Sarcoma 180) explanted to the chorioallantoic membrane of the chick embryo. To be published.
12. Karnofsky, D. A. 1955. The use of the developing chick embryo in pharmacologic research. Stanford Med. Bull. **13**: 247.
13. Karnofsky, D. A. 1955. Assay of chemotherapeutic agents on the developing chick embryo. *In* Investigation of Diverse Systems for Cancer Chemotherapy Screening. Cancer Research. **3** (Suppl.): 83.
14. Stock, C. C., J. J. Biesele, J. H. Burchenal, D. A. Karnofsky, A. E. Moore & K. Sugiura. 1950. Folic acid analogs and experimental tumors. Ann. N. Y. Acad. Sci. **52**(8): 1360.
15. Dagg, C. P., D. A. Karnofsky, H. W. Toolan & J. A. Roddy. 1954. Serial passage of human tumors in chick embryo; growth inhibition by nitrogen mustard. Proc. Soc. Exptl. Biol. Med. **87**: 223.
16. Bodenstein, D. 1949. The induction of aristapedia by nitrogen mustard in *Drosophila virilis*. J. Exptl. Zool. **111**: 95.
17. Schultz, J., N. Rothman & M. M. Aronson. 1955. The growth and morphogenesis of *Drosophila melanogaster* as criteria for screening tests. *In* Investigation of Diverse Systems for Cancer Chemotherapy Screening. Cancer Research. **3** (Suppl.): 86.
18. Bodenstein, D. 1948. The effects of nitrogen mustard on embryonic amphibian development. II. Effects on eye development. J. Exptl. Zool. **108**: 93.
19. Danforth, C. H. & E. Center. 1954. Nitrogen mustards as a teratogenic agent in the mouse. Proc. Soc. Exptl. Biol. Med. **86**: 705.
20. Didcock, K., D. Jackson & J. M. Robson. 1956. The action of some nucleotoxic substances on pregnancy. Brit. J. Pharmacol. **11**: 437.
21. Thiersch, J. 1957. Effects of 2,4,6 triamino-"s"-triazine (TR), 2,4,6 "tris"-(ethyleneimino)-"s"-triazine (TEM) and N,N',N''-triethylene phosphoramide (TEPA) on rat litter in utero. Proc. Soc. Exptl. Biol. Med. **94**: 36.
22. Hicks, S. P. 1953. Developmental malformations produced by radiation. Am. J. Roentgenol. **69**(2): 272.
23. Bodenstein, D. & A. Goldin. 1948. A comparison of the effects of various nitrogen mustard compounds on embryonic cells. J. Exptl. Zool. **108**: 75.

Discussion

J. Bennet Olson (*Children's Hospital, Los Angeles, Calif.*): I wish to compliment Lois Murphy on the careful planning and execution of her experimental studies. Working quite independently and from a different point of departure, Sidney S. Sobin and I have achieved results with TEM that confirm the results Murphy has reported. Our chief concern, however, was the production of congenital defects of the cardiovascular system. If Murphy had had the opportunity of examining the hearts of her specimens, I am sure she would have found that the most common defects produced by her alkylating agents were not external but, rather, defects of the heart. We found that dosages that did not produce external defects in any significant numbers did produce defects of the ventricular septum of the rat heart. Higher doses increased the incidence of cardiovascular defects as well as the external defects described in this monograph. It is not surprising that the heart is more sensitive to teratogenic agents when one considers the complexity of its embryonic history. There are so many things that can go wrong with its normal pattern of development that one would expect teratogenic agents easily to upset the normal course of events at some point or other in its development. Embryologists have paid too little attention to the heart in reporting the effects of such agents on other organs of the vertebrate body. In the areas in which our studies overlap those of Murphy, they are complementary. Where they do not, our studies are supplementary to hers. Comparisons of the two will be of interest.

COMPARATIVE EFFECTS OF ALKYLATING AGENTS ON CELLULAR MORPHOLOGY*

By P. C. Koller
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London, England*

At the outbreak of World War II, investigation was organized in various research centers to study the biological effects of vesicant poison gases such as lewisite and mustard gas in order to find antidotes. A major development in this direction was Auerbach's discovery (1943), using genetic methods, that an extremely minute dose of sulfur mustard ($\beta\beta'$-dichlorodiethylsulfide) induced gene mutations, that is, permanent changes in the nuclear component of cells. Her finding has shown that the lethal effect of this vesicant on cells is nucleotoxic instead of cytoplasmic, as was believed (Auerbach *et al.*, 1947).

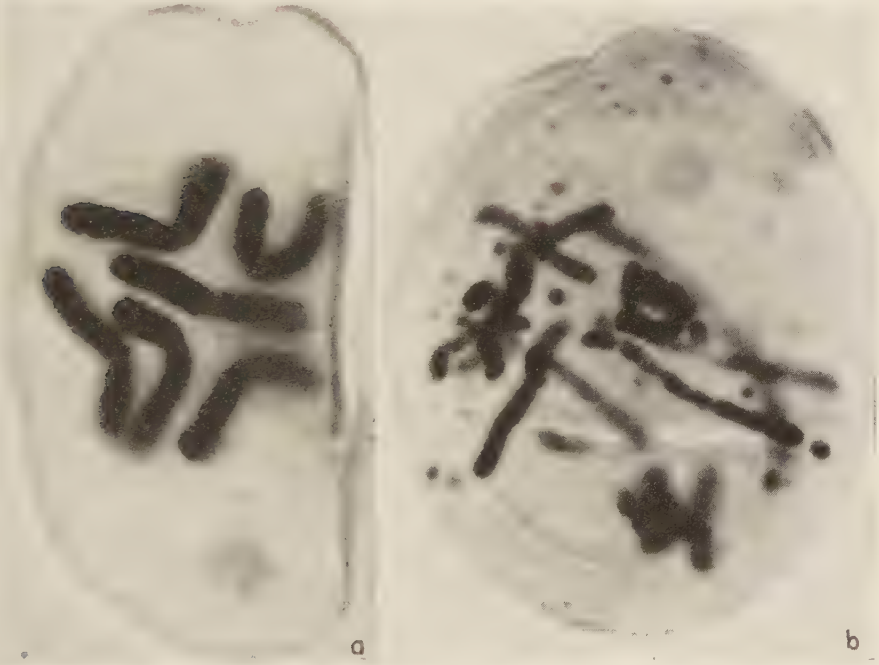

FIGURE 1. Pollen grain of *Tradescantia:* (a) metaphase of mitosis, showing the six chromosomes, and (b) chromosome fragmentation induced by aerosol of sulfur mustard. × 3200.

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* The work reported in this paper was supported by grants to the Chester Beatty Research Institute from the British Empire Cancer Campaign, London, England; the Jane Coffin Childs Memorial Fund for Medical Research, New York, N. Y.; the Anna Fuller Fund, New Haven, Conn.; and the National Cancer Institute, Public Health Service, Bethesda, Md.

In view of the great importance of Auerbach's discovery, I undertook the study of the *direct* effects of mustard gas and two other selected vesicants, di- and tri-chloroethylamines, on the chromosomes of pollen grains of

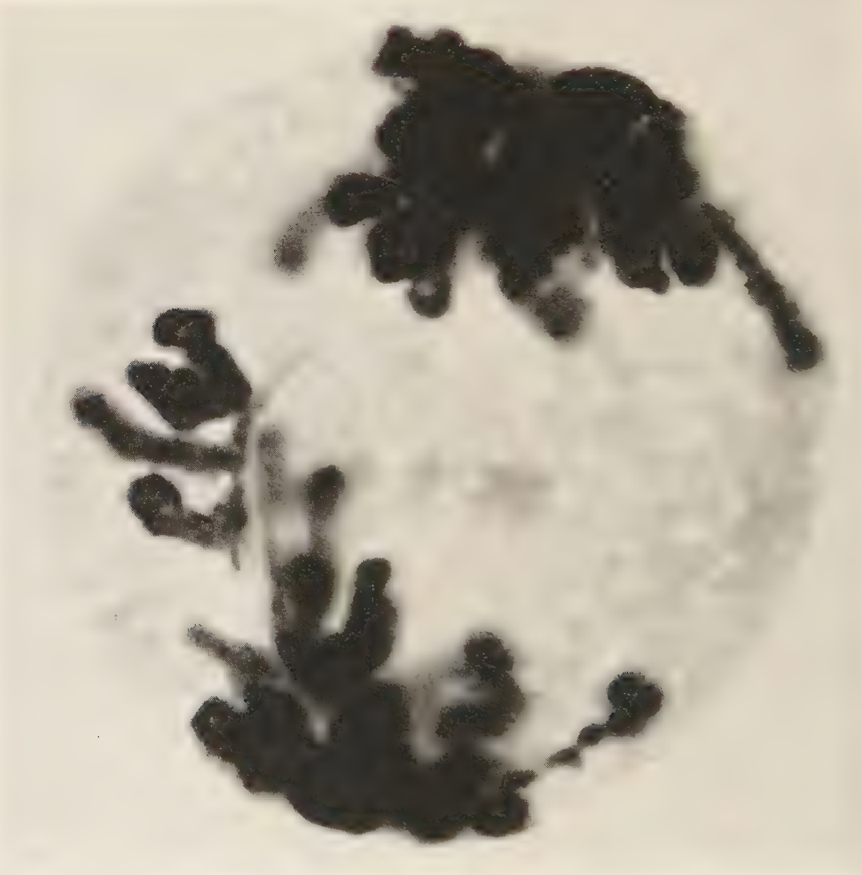


FIGURE 2. Misdivision or transverse split of the centromere during meiosis, resulting in the fragmentation of chromatid arms (aerosol of sulfur mustard, *Tradescantia* pollen mother cell). × 4000.

Tradescantia. Evidence supporting Auerbach was obtained (FIGURE 1, Koller, 1947).

Some of the observations made on the response of plant chromosomes to the aliphatic mustards may be summarized as follows:

(1) The centromere or dynamic center of meiotic and mitotic chromosomes is particularly sensitive. Misdivision, that is, the transverse split of the centromere, occurs frequently (FIGURE 2).

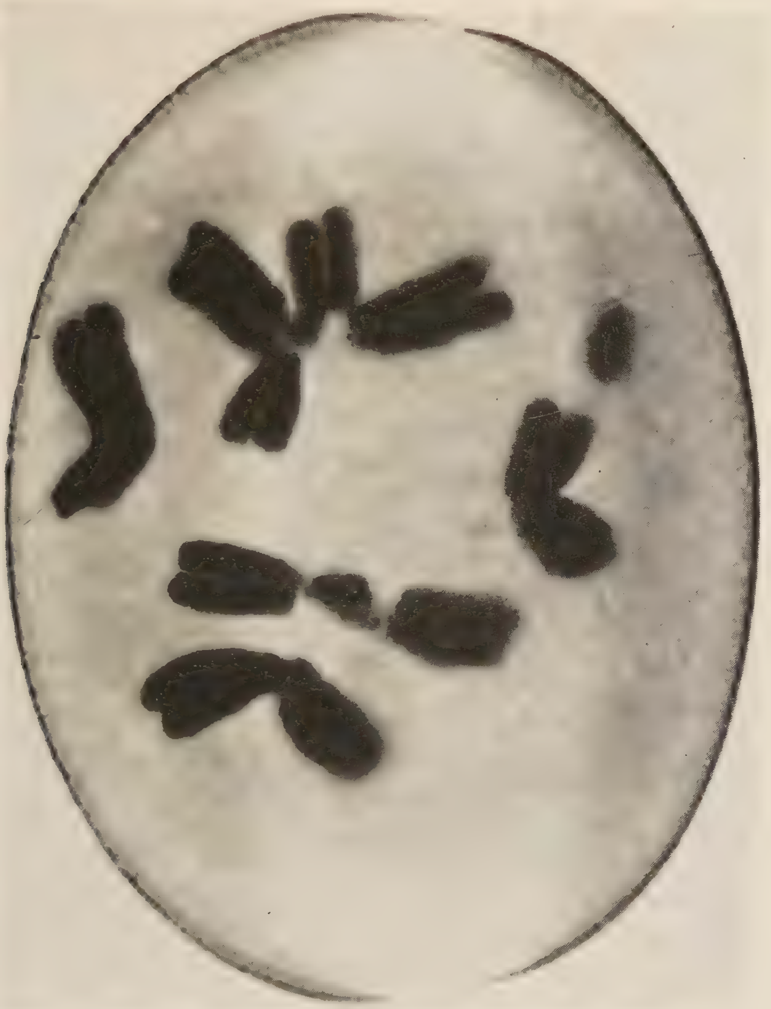


FIGURE 3. Chromatid interchange between two chromosomes occurring at the centromere. The other chromosomes show fragmentation (aerosol of sulfur mustard, *Tradescantia* pollen grain). × 4000.

(2) Breakage of chromatids takes place with a high frequency at or near the centromere. The breaks are usually followed by chromatid interchanges (FIGURE 3).

(3) Exposure to these drugs results in a stickiness of chromosomes that produces pseudochiasmata.

(4) Despiralization of chromosome regions indicates that the coiling mechanism of the nucleoprotein chromosome fiber is affected by the aliphatic mustards (FIGURE 4).

Since 1946 I have been able to study and to compare cell behavior in mammalian tissues that have been exposed to a variety of both aliphatic and aromatic mustards, most of which were synthesized in the Chester Beatty Research Institute. These investigations have disclosed that the cytological effects, particularly the injuries to the chromosome mechanism of cells that appear after the administration of these agents are very similar to those induced by irradiation. Because of these and other similarities, they are frequently referred to as "radiomimetic" drugs (Dustin, 1947).

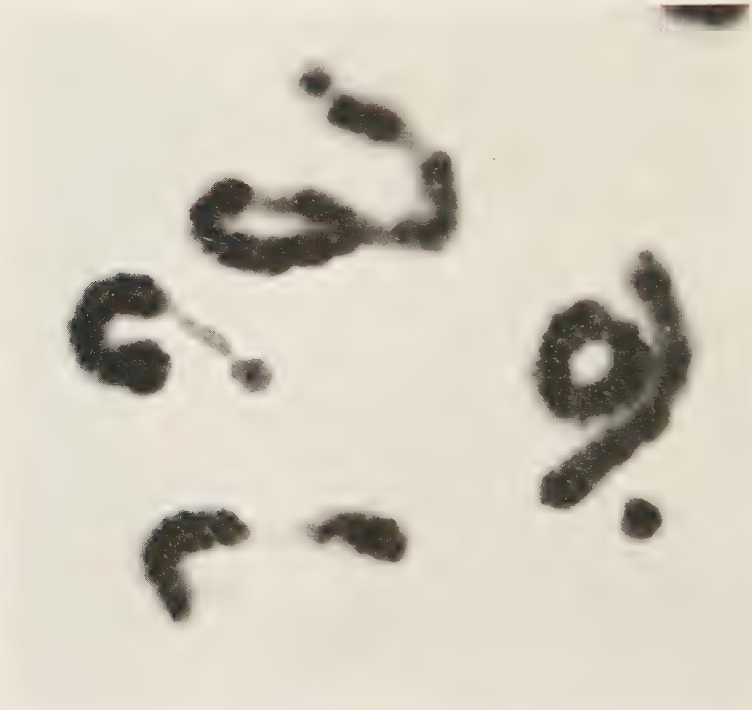


FIGURE 4. Despiralization of chromosome segments induced during meiosis of aerosol of sulfur mustard (*Tradescantia* pollen mother cell). × 4000.

Quantitative analysis of the cytological effects of the nitrogen mustard (HN2) was carried out on the cells of the transplantable Walker carcinoma 256 of the rat. The data are given in TABLE 1. FIGURE 5 shows the frequency distribution of injured cells in the Walker carcinoma after HN2 and X radiation (Koller and Casarini, 1952). The frequency of cells with chromosome injuries, observed at different times after exposure to X rays, has been used to identify the stage at which the cell is most sensitive to ionizing radiation. The same method may be used to determine the stage at which it is most sensitive to drugs. The reliability of this procedure, however, depends on various conditions, for example: (1) the transportation of the drug to the site of action must be rapid; (2) the activity of the drug must be of short duration within the cell; and (3) the rate of development of the cell must not be altered by the drug. It was believed that the rapidly hydrolyzing HN2 fulfilled these conditions, and the comparison of the cytological effects after X rays and HN2 seemed to indicate a difference in the stage during which the cell was most responsive to these agents. However, when the types of cell injuries were analyzed, it became obvious that they are of two kinds. Those that appear not later than 48 hr. after one

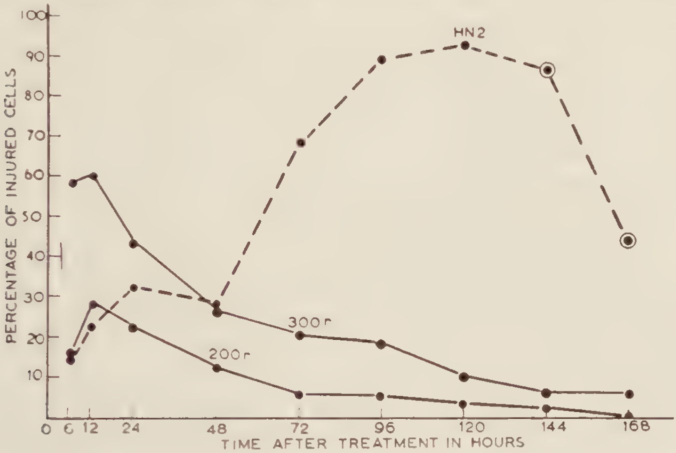


Figure 5. Frequency distribution of injured tumor cells in Walker carcinoma 256 after X rays and nitrogen mustard (HN2).

Table 1

Frequency of Injured Tumor Cells in the Walker Carcinoma After the Administration of Nitrogen Mustard (HN2)*

Time after injection (hours)	No. of cells in post-metaphase	No. of cells with chromosome injuries	
		Number	Per cent
6	50	7	14
12	50	11	22
24	50	16	32
48	50	14	28
72	77	42†	54.7
96	53	48†	90.5
120	34	32†	94.0
144	42	37†	88.0
168	18	8†	44.7

* Intraperitoneal injection of 1 mg./kg.

† Most of the cells had shown "superfragmentation," an excessive breakage of chromosomes.

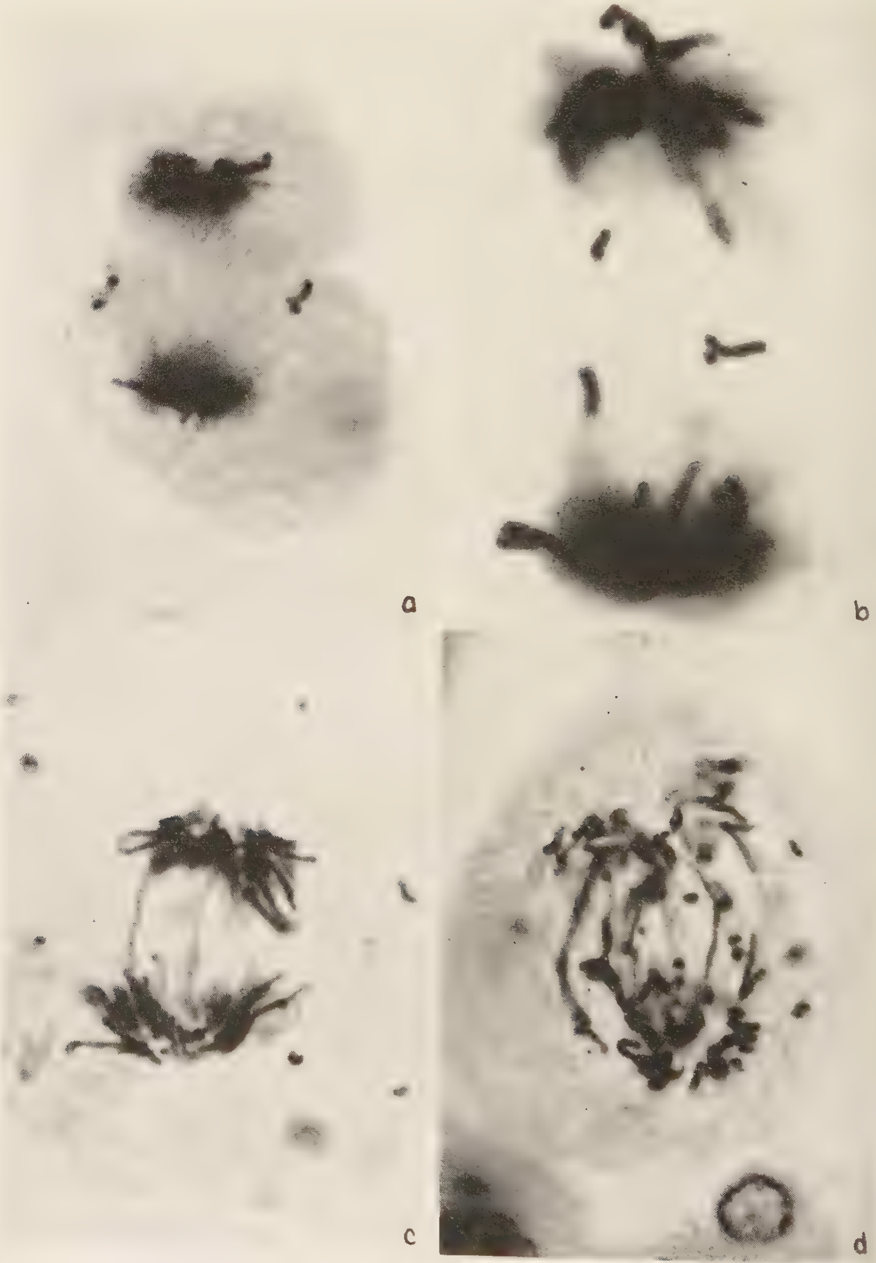


FIGURE 6. True radiomimetic (or nucleotoxic) effects: (a and b) dividing tumor cells in anaphase, showing the "acentric" or inert chromosome fragments, induced 24 hr. after treatment with HN2 (Walker carcinoma 256, 1 mg./kg. I.P.); (c) dividing tumor cell with chromosome bridges and fragments that appear 48 hr. after treatment with HN2 (Walker carcinoma 256, 1 mg./kg. I.P.); and (d) chromosome bridges and fragments in a dividing tumor cell of Walker carcinoma, 56 hr. after HN2 (1 mg./kg. I.P.). × 2500.

single intraperitoneal injection of the drug are true radiomimetic (or nucleotoxic) effects; that is, they consist of chromosome breaks and bridges (FIGURE 6). On the other hand, the few dividing cells of tumor tissue found 72 hr. after the administration of the drug exhibit gross injuries to their chromosome mechanism (FIGURE 7). It seems that the chromosomes have undergone excessive fragmentation; the fragments are scattered in the cytoplasm and show various degrees of abnormal condensation (pyknosis). The injury is too serious to the cell and, because the chromosome mechanism is destroyed, the cell fails to complete mitosis. The same type of injury can be seen after irradiation only when a tissue is exposed to heavy doses (5000 to 9000 r). In the latter case it was discovered that the heavily damaged cells were delayed from entering mitosis. It is very likely that cells showing "superfragmentation" 72 hr. after HN2 treatment have been similarly delayed.

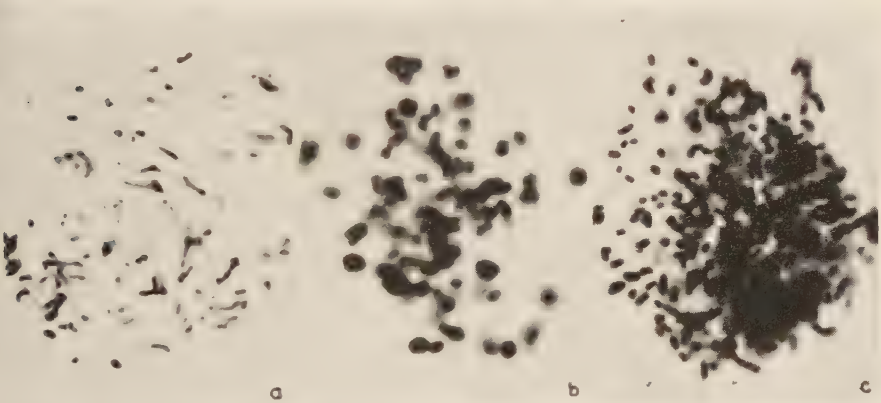


FIGURE 7. Dividing tumor cells, showing various degrees of superfragmentation of the chromosome mechanism. Walker carcinoma 256: (a) 72 hr. after administration of 1,2-3,4-diepoxybutane; (b) HN2; and (c) an aromatic nitrogen mustard. × 1800.

It is also probable that the excessive fragmentation of the chromosomes may be the result of gross metabolic disturbance induced in the cell by the drug (Koller, 1955). In this case the superfragmentation of chromosomes is an indirect injury and differs from the true radiomimetic injuries. In order to distinguish the two types, superfragmentation may be described as a cytotoxic effect. For these reasons, therefore, it can be assumed that the abnormal cells observed 72 hr. after the administration of HN2 represent a heterogeneous group that contains cells with true radiomimetic effects as well as cells with cytotoxic damage. On that account, only with reservations can the frequency distribution of cytological injuries due to HN2 be used for comparison with those due to X rays.

The Spectrum of Cytological Injuries and Growth Inhibition

Cytological analysis of Walker carcinoma after irradiation has shown that mitotic injuries are important effects that cause permanent or temporary inhibition of growth. Other investigators and I (Koller and Casarini, 1952; Hendry *et al.*, 1951) obtained similar findings when the tumor-bearing animals were injected with certain alkylating agents. One of the most important factors in bringing about growth inhibition is the number of injured cells in mitosis and the length of time that elapses between administration of the drug and the peak frequency of cellular injury. By increasing the dose the number of injured cells can be increased, and the time when the "peak" injury appears after treatment can be altered. TABLE 2 shows the frequency of abnormally dividing cells after a single intraperitoneal administration of a sulfonic acid ester at three different dosages. It can be seen that the time when the highest number of dividing cells show chromosome injuries shifted from 48 to 72 hr. after the dose had been increased from 4 to 8 mg./kg.

TABLE 2

FREQUENCY OF CELLS WITH MITOTIC INJURIES IN THE WALKER CARCINOMA AFTER VARIOUS DOSES OF CB 2041

Dose (I.P. inject.)	Time after treatment (hours)				Growth inhibition
	24	48	72	96	
4 mg./kg.	17(100)*	28(97)	10(100)	5(88)†	Nil
6 mg./kg.	24(100)	40(100)	31(91)	14(82)	Temporary
8 mg./kg.	37(100)	71(102)‡	83(100)‡	35(55)‡	Permanent

* The numbers in brackets indicate the total number of dividing cells analyzed.

† The frequency of spontaneous mitotic abnormality was 5 per cent in the Walker carcinoma.

‡ A great proportion of injured cells had cytotoxic abnormalities (superfragmentation).

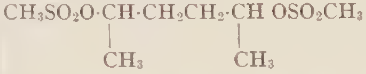
The three types of cytological injury—(1) suppression of mitosis, and (2) radiomimetic and (3) cytotoxic effects—and their importance in growth inhibition—have been studied in the Walker carcinoma after the administration of a series of aliphatic sulfonic acid esters. In TABLE 3 the activity of 18 members of the aliphatic sulfonic acid esters are summarized. These drugs were synthesized by G. Timmis at our institute (Haddow and Timmis, 1951).

It was found that there is a period, commencing 12 to 18 hr. after administration of the drugs, during which the number of dividing cells significantly decreases and mitosis may disappear completely from the tumor. The onset and duration of the mitosis-free period depends chiefly on the molecular constitution and dose of the drug—the higher the dose, the earlier the onset and the longer the duration of mitosis suppression.

The dose of each compound shown in TABLE 3 was determined by the criteria that it produces injuries in at least 25 per cent of dividing tumor cells and that the peak period of injury falls within 72 hours after adminis-

TABLE 3

TYPES OF CYTOLOGICAL INJURIES INDUCED BY VARIOUS SULFONIC ACID ESTERS IN THE WALKER CARCINOMA

Compounds				Cytological injuries*			Inhibition of growth	
Serial number	Molecular structure	Code number	Dose (kg., body wt.; I.P. injection)	A	B	C	Temporary	Permanent
I	$CH_3SO_2O(CH_2)_2OSO_2CH_3$	CB 2039	125 mg.	−	⊕	⊕	−	−
II	$CH_3SO_2O(CH_2)_3OSO_2CH_3$	2040	100 mg.	+	+	+	+	−
III	$CH_3SO_2O(CH_2)_4OSO_2CH_3$	2041	8 mg.	+	+	+	−	+
IV	$CH_3SO_2O(CH_2)_5OSO_2CH_3$	2064	15 mg.	−	−	+	+	−
V	$CH_3SO_2O(CH_2)_6OSO_2CH_3$	2050	50 mg.	+	+	+	−	−
VI	$CH_3SO_2O(CH_2)_7OSO_2CH_3$	2101	50 mg.	+	+	+	+	−
VII	$CH_3SO_2O(CH_2)_8OSO_2CH_3$	2078	60 mg.	+	+	+	+	−
VIII	$CH_3SO_2O(CH_2)_9OSO_2CH_3$	2067	40 mg.	+	−	+	+	−
IX	$CH_3SO_2O(CH_2)_{10}OSO_2CH_3$	2052	125 mg.	+	−	+	−	−
X	$CH_3SO_2O(CH_2)_{18}OSO_2CH_3$	2086	150 mg.	−	−	⊕	−	−
XI	$CH_3SO_2OCH_2CH_3O\ CH_2CH_2OSO_2CH_3$	2020	125 mg.	−	+	+	−	−
XII	$CH_3SO_2OCH_2C{\equiv}C\ CH_2OSO_2CH_3$	2058	15 mg.	−	+	+	+	−
XIII	*p*-$CH_3SO_2O\ (CH_2)_2C_6H_4(CH_2)_2OSO_2CH_3$	2100	50 mg.	−	⊕	⊕	−	−
XIV	*o*-$CH_3SO_2O\ (CH_2)_2C_6H_4(CH_2)_2OSO_2CH_3$	2093	50 mg.	+	⊕	⊕	−	−
XV	*cis*-$CH_3SO_2OCH_2CH{=}CH\ CH_2OSO_2CH_3$	2094	5 mg.	+	−	+	−	−
XVI	*trans*-$CH_3SO_2OCH_2CH{=}CH\ CH_2OSO_2CH_3$	2095	20 mg.	−	⊕	+	+	−
XVII	$CH{\equiv}CCH_2O{\cdot}SO_2CH_3$	2116	200 mg.	−	−	−	−	−
XVIII	 $CH_3SO_2O{\cdot}\underset{CH_3}{CH}{\cdot}CH_2CH_2{\cdot}\underset{CH_3}{CH}\ OSO_2CH_3$	2348	5 mg.	−	+	⊕	−	−

* A = mitotic suppression; B = radiomimetic effects; C = superfragmentation, cytotoxic effects; ⊕ = frequency of injured cells is less than 10 per cent (frequency of "spontaneous" mitotic abnormality is 5 per cent in Walker carcinoma).

tration of the drugs. Compounds I, X, XIII, XIV, and XVI induced damage in less than 10 per cent of the tumor cells, and the number of injured cells could not be increased because of the serious toxic effects that resulted in the death of the animals. TABLE 3 also shows that certain cellular injuries are absent after the administration of particular compounds; for example, CB 2039 (I) and CB 2064 (IV) failed to suppress mitosis. The latter also failed to produce radiomimetic chromosome injuries. CB 2116 (XVII) was inactive in spite of the very high dose injected. CB 2086 (X) produced only mitotic pyknosis due to superfragmentation of chromosomes, without suppressing mitosis or inducing chromosome injuries of the true radiomimetic type.

The highest activity was shown by CB 2041 (III); all stages of cellular injury were observed after the administration of this drug, which could inhibit also the growth of well-established Walker tumors. In previous studies I had found that, in order to suppress the growth of 5- to 7-day-old, rapidly growing Walker carcinoma, it was necessary to injure not less than 70 to 90 per cent of dividing tumor cells and that the maximum injury must be produced not later than 48 to 72 hr. after administration of the drug or X rays (Koller and Casarini, 1952; Koller, 1955). CB 2041 (III) seems to satisfy these requirements.

The growth-inhibiting property of CB 2041 was established by other workers using various tumors. It was found that this particular aliphatic sulfonic acid ester retarded the growth of an adenocarcinoma and carcinoma 1025, mammary carcinoma 755, glioma 26 in mice, and Brown-Pearce carcinoma in rabbits (Gellhorn *et al.*, 1955; Sugiura, 1955).

An interesting pattern of response was found after CB 2064 (IV) had been given; it does not suppress mitosis, and produces only gross chromosome injuries, which causes cell degeneration. The cytological analysis suggested also that the activity of the *cis*- and *trans*-isomers (CB 2094 and 2095, respectively) of 1,4-dimethanesulfonoxy-2-butane may differ. It was found that, while the cell injuries induced by CB 2095 lead to temporary arrest of Walker carcinoma, growth was not inhibited when the *cis*-isomer (CB 2094) was used. The toxicity of the latter is about 4 times greater than that of the former.

Molecular Constitution and Biological Activity

The cytological effect of a mustard derivative of the natural amino acid of phenylalanine (CB 3007) has been analyzed recently. The drug and its two isomeric forms (CB 3025 and CB 3026) have been synthesized by Bergel and Stock (1954).

The cytological injuries observed in the tumor cells after administration of the two isomeric forms of this drug were similar to those seen after administration of aromatic or aliphatic mustards (Koller and Veronesi, 1957). Biological tests have shown that this compound is more active as a growth inhibitor of the Walker carcinoma in the L form than in the D or DL form. Similarly, a comparison of survival time of mice with Landschütz ascites has also indicated the greater efficiency of the L isomer. When the number

TABLE 4

FREQUENCY OF INJURED CELLS IN THE WALKER CARCINOMA AND LANDSCHÜTZ ASCITES TREATED WITH THE ISOMERS OF THE MUSTARD DERIVATIVE OF PHENYLALANINE*

Tumor	Dose	Isomeric form	Per cent of cells with mitotic injuries					
			24 Hr.	48 Hr.	72 Hr.	96 Hr.	120 Hr.	144 Hr.
Walker carcinoma (rat)	1 mg./kg.	L	40	61	48	—	—	—
		D	34	52	40	—	—	—
		DL	27	53	42	—	—	—
Landschütz ascites (mouse)	8 mg./kg.	L	64	85	81	76	—	—
		D	55	71	76	78	—	—
	4 mg./kg.	L	45	69	72	76	71	60
		D	42	79	67	61	66	51
		DL	40	73	—	—	46	65

* One hundred dividing cells were counted in each cell.

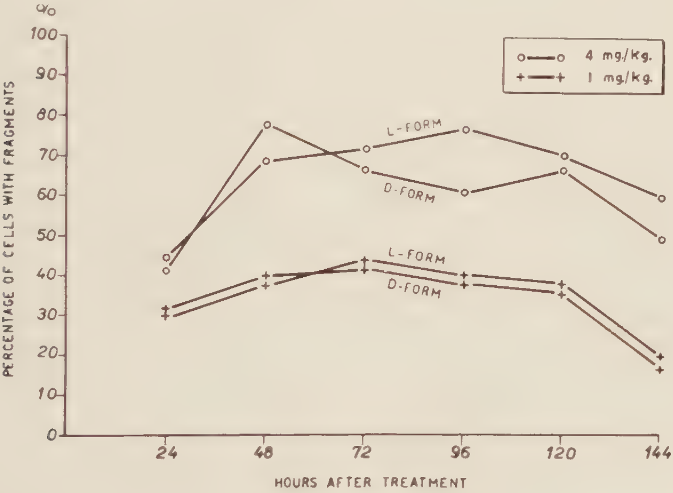


FIGURE 8. Frequency distribution of dividing cells with chromosome injuries in the Landschütz ascites after treatment with a nitrogen mustard derivative of phenylalanine amino acid.

of cells with mitotic injuries was used as the basis of comparison, however, it was found that the difference between the two isomers is not very significant (TABLE 4; FIGURE 8).

This observation suggests strongly that growth inhibition is not brought about by the death of tumor cells due only to chromosome injuries; it is more likely that other events, for example, suppression of mitosis, cell pyknosis in the resting stage, and stromal reaction, which follow the administration of the drugs, also played a role in growth suppression.

The cytological effects of CB 3026 (L form) have been analyzed in the Walker and "August" carcinoma and "Yoshida" sarcoma in rats and in three mouse ascites tumors. TABLE 5 shows the number of injured cells observed in these tumors 24 and 48 hr. after a single intraperitoneal injection of CB 3026 (L form). The drug failed to inhibit growth of the August tumor and the Yoshida sarcoma. Although the histological structure of both of these tumors differs from that of the Walker carcinoma, the failure of growth inhibition cannot be attributed to that difference alone. The fact that Yoshida sarcoma shows the same response to CB 3026, either as an ascites or solid subcutaneous growth, suggests that the difference is due to the inherent nature of the malignant cells of these tumors.

Selectivity of Drug Action

In the therapy of cancer the aim is to arrest the cell multiplication responsible for malignant growth. Injury to the mitotic mechanism is one way by which cell multiplication can be stopped. It appears that the alkylating agents are highly effective in producing mitotic disturbances or nucleotoxic effects that result in the death of the injured cell. Experience indicates also that not only the tumor tissue, but other tissues in which cell multiplication takes place at a high rate, for example, bone marrow, intestinal mucosa, testes, and ovaries, are damaged by these agents.

TABLE 5

FREQUENCY OF INJURED CELLS OBSERVED IN DIFFERENT TUMORS AFTER THE INJECTION OF THE MUSTARD DERIVATIVE OF PHENYLALANINE (L ISOMER)*

Animal	Tumor	Dose (I.P. injection)	Per cent of cells injured	
			24 Hr.	48 Hr.
Rat	Walker carcinoma (solid)	1 mg./kg.	40	61
	Walker carcinoma (ascites)	0.25 mg./kg.	32	51
	August carcinoma	1 mg./kg.	7	—
	Yoshida carcinoma (solid)	1 mg./kg.	10	—
	Yoshida sarcoma (ascites)	0.50 mg./kg.	13	7
Mice	Krebs-2 (ascites)	2 mg./kg.	19	12
	Ehrlich (ascites)	2 mg./kg.	23	31
	Landschütz (solid)	2 mg./kg.	16	12
	Landschütz (ascites)	2 mg./kg.	30	38

* One hundred dividing cells were counted in each tumor.

I have observed the damaging effects of several alkylating agents in the testes and, particularly, in the bone marrow. Nearly all the agents studied produced toxic reactions in the femoral marrow of rats and mice. The giant multinucleate megakaryocytes of the rat are the first cells to be injured.

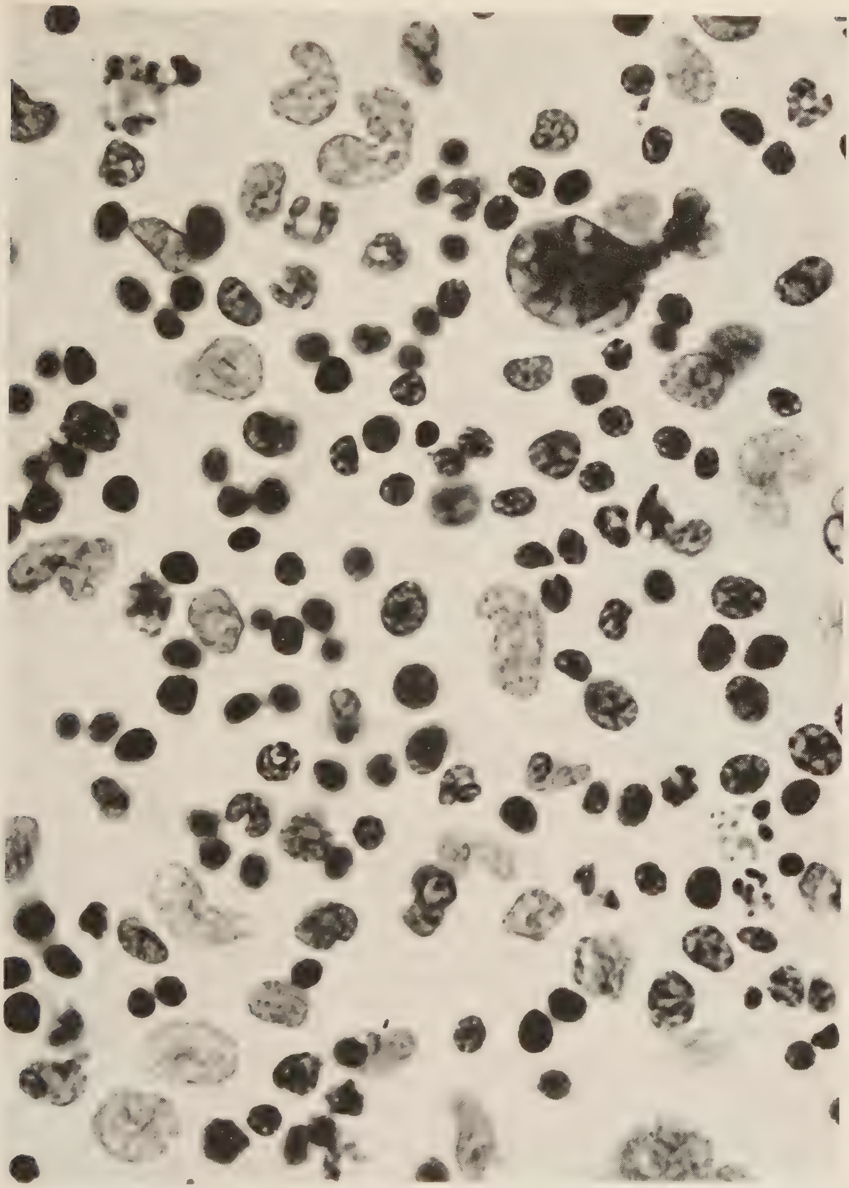


FIGURE 9. Pyknotic degeneration of megakaryocyte in the femoral marrow of a rat 20 hr. after treatment with the aliphatic sulfonic acid ester CB 2050 (TABLE 3). × 750.

It has been observed that the numerous nuclei of these giant cells condense and "clump" together into a deeply staining pyknotic body (FIGURE 9). Megakaryocytes are extremely sensitive to toxic substances and can undergo pyknotic degeneration as early as 10 hr. after administration of particular drugs such as nitrogen mustard and certain sulfonic acid esters.

The damage to megakaryocytes is followed closely by the appearance of a very large number of lymphocytes and reticulum cells that drastically change the cellular composition of the hemopoietic marrow. The influx of lymphocytes may represent an inflammatory response. Abnormalities in cells of the myeloid series begin to appear at about 40 to 48 hr. after administration of the drug. It was observed that the nuclei of myelocytes and myeloblasts increase in size and that their intranuclear network becomes diffuse. Dividing cells with few chromosome fragments and bridges may also appear at this time. Similar changes were seen in cells of the erythroid series. Cells with several deeply stained pyknotic bodies of varying sizes are found frequently in the marrow 48 hr. after treatment. These are assumed to be degenerating cells in which the mitotic mechanism has been greatly injured, and in which the fragmented chromosome segments have undergone pyknotic condensation in the cytoplasm.

As a result of extensive degeneration of the myeloid and erythroid elements, the cell population in the marrow is composed mostly of reticulocytes, undifferentiated mesenchymal cells, and monocytes. The reorganization and repopulation of the damaged bone marrow with myeloid and erythroid cells is a gradual and slow process and may require between 4 and 8 weeks to restore the marrow to normal.

It has been found that one member of the sulfonic acid esters, CB 2041, had a particularly depressant action on the neutrophils in the circulating blood of the rat (Haddow and Timmis, 1953). For this reason, I have undertaken studies of the cellular reactions that occur in rat marrow after the administration of this particular compound in the hope of throwing some light on the cytological basis of the neutrophil depressant action.

The cytological studies disclosed that the maturation and release of granulocytes into circulation is delayed by CB 2041. No such effect was produced on the erythroid cells. The most interesting phenomenon, however, was the breakup of the polymorphonuclear cells due to fragmentation of their segmented nuclei (FIGURE 10). The types of injury produced in the well-differentiated granulocytes must be distinguished from the radiomimetic effects produced by the same drug in dividing cells. It is obvious that the destruction of mature granulocytes by CB 2041 was brought about without mitotic injuries. Selective action on the granulocytes led to the introduction of CB 2041, under the name Myleran (Haddow and Timmis, 1953; Galton, 1953), into the therapy of myeloid leukemia.

In view of the selective action of Myleran on particular cell types, it should be emphasized that the ability of the alkylating agents to produce radiomimetic injuries in cells is not necessarily the most important property that determines the effectiveness of these drugs in cancer therapy. Thus, the nitrogen mustard, producing both gene and chromosome mutation in many animals and plants, is a typical radiomimetic poison. However, the cytological studies of bone marrow of human patients show that the therapeutic value of HN2 in lymphoid leukemia and lymphosarcoma is not wholly due to its radiomimetic properties.

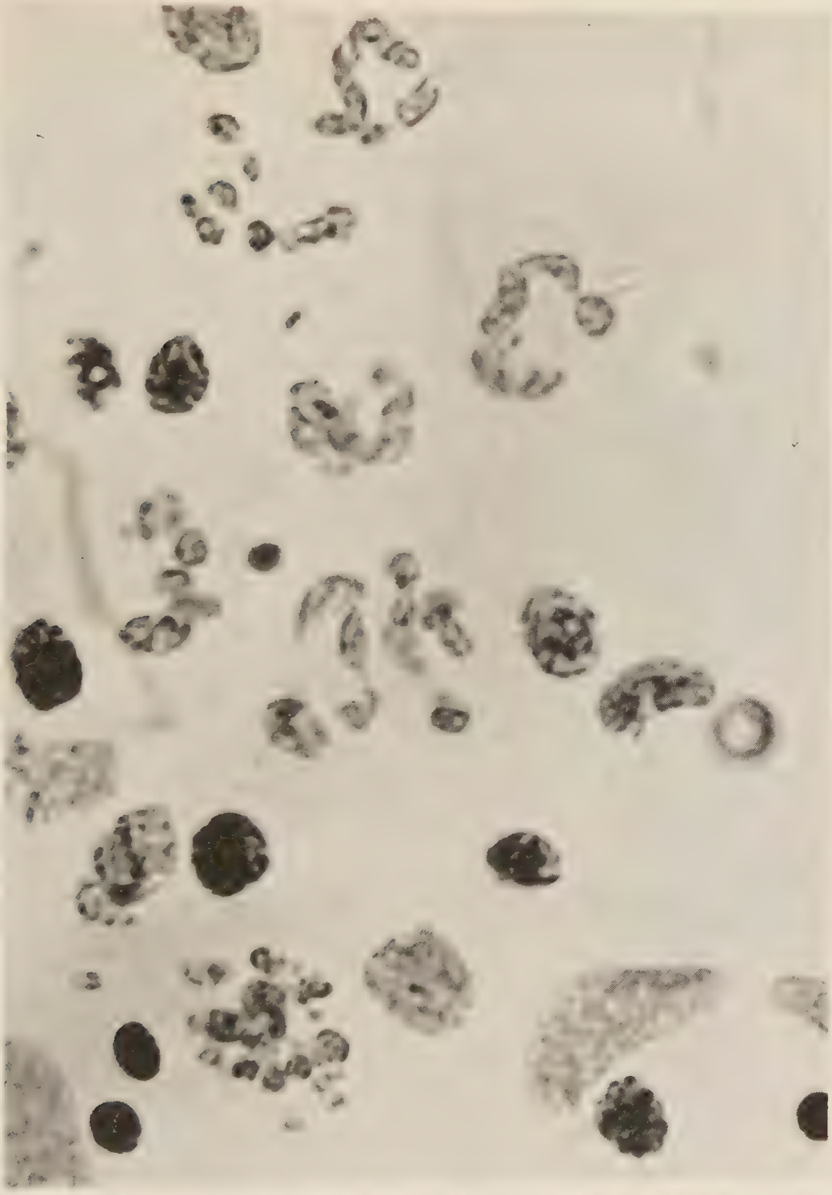


FIGURE 10. Nuclear fragmentation in mature granulocytes of the rat 72 hr. after treatment with the aliphatic sulfonic acid ester CB 2041 (TABLE 3). × 750.

Cytological Peculiarities in Tumors Induced by Alkylating Agents

Many tumors have been produced by the various alkylating agents in rats and mice, usually at the site of subcutaneous injection. The cytological behavior was studied in 81 primary tumors, mostly sarcomata, induced by aliphatic and aromatic mustards, epoxides, triethylene melamine, and sulfonic acid esters. It was found that, in the different primary tumors induced by the same agent, the type and incidence of chromosome abnormalities very often differed. In some tumors no abnormal mitoses were seen while, in others, all types were represented with a high frequency.

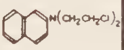
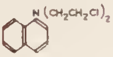
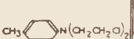
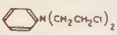
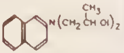
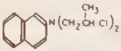
Compound	*Tumor*	*No. of Transplantation*	*Cytological features*			*Notes*	PER CENT OF MITOTIC ABNORMALITIES
			Anaphase Bridges	*Fragments*	*Polyploidy*		
CB 1048 	A	93 Gen	+ + +	+	+	*Permanent*	16·8
	B	10	+	+	+	*Temporary*	5·6
	C	7	−	−	+ +	*Temporary*	8·0
	D	0	−	+	−		NOT ANALYZED
	E	2	+ +	+	+	*Temporary*	6·0
	F	8	−	−	−	*Normal*	NIL
	G	3	+	+	+	*Semipermanent*	12·6
CB 1047 	A	0	−	+	+		3·2
	B	0	−	−	+ +		9·5
	C	0	−	−	−		NIL
	D	7	+	−	+	*Temporary*	3·0
	E	4	+	+ +	+	*Temporary*	4·5
	F	0	−	+	+		7·5
CB 1044 	A	0	+ +	−	−	"Localized" chromosome stickiness	18·0
	B	0	−	+	+		2·0
	C	2	+	+	−	*Temporary*	NOT ANALYZED
	D	0	−	−	−		NIL
	E	2	−	−	+		7·0
CB 1074 	A	8	−	−	+	*Temporary*	5·7
	B	2	−	−	+ +		12·0
	C	0	−	+	−		7·0
CB 1075  ISOMER M.P. 100°	A	0	−	−	+		3·6
	B	0	−	−	−		11·0
	C	0	+	+	+ +		7·0
	D	0	−	+	+ +	TREATED WITH *ACTH*	NOT ANALYZED
	E	3	+	−	+ +		6·0
	F	0	−	+	−	*"Blister in tumor" contained RTS*	10·0
	G	0	+	+	+	*"Blisters"*	22·5
	H	0	+	+	+		13·0
CB 1076  ISOMER M.P. 83°	A	0	−	+	+		NOT ANALYZED
	B	0	−	−	−		3·2
	C	0	−	+	+ +	*"Blisters"*	7·5
	D	3	+	−	+	*Semipermanent*	3·0
	E	0	−	−	+		5·2
	F	0	+	+	+ +		14·0

FIGURE 11. Cytological characteristics of nitrogen-mustard-induced tumors in the rat.

FIGURE 11 illustrates the cytological features of some primary tumors induced by aromatic mustards. It is interesting to note that most of the abnormalities were only temporary, and that they were rarely seen in transplanted tumors derived from primary tumors rich in abnormally dividing cells. The exceptions are polyploidy and dicentric chromosomes that appeared in a tumor induced by a particular aromatic mustard; these were maintained in more than a hundred transplant generations (FIGURE 12; Koller, 1953).

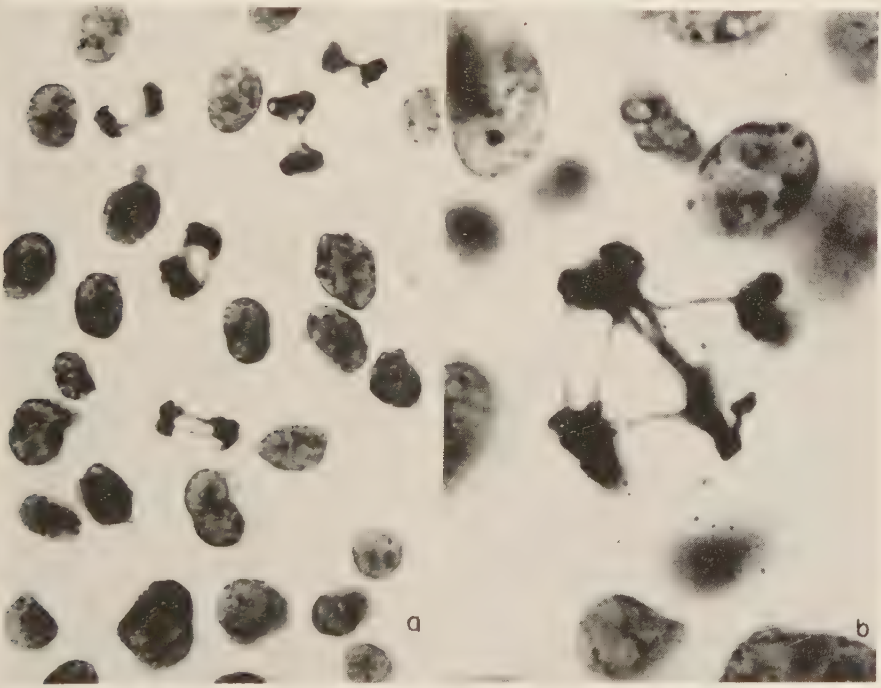


FIGURE 12. Dividing cells with chromosome bridges in a primary tumor induced by the aromatic nitrogen mustard CB 1048 (FIGURE 11). (b) × 1500.

The question naturally arises: What is the cause of the temporary chromosome anomalies present in almost every primary tumor? It is very likely that the cause of the mitotic abnormalities is inherent in the process responsible for the establishment of malignant growth. By analyzing the early development of tumors, I obtained evidence showing that, in addition to the reaction by the host, the rapid multiplication of malignant cells and the lack of histological organization create an environment in which tumor cells are selected. This process is extremely rigorous in the primary growth and is reflected in the high number of mitotic abnormalities. Only those cells that adapt themselves to the environment will be able to multiply.

It is also possible that the carcinogenic drug itself was responsible for the mitotic abnormality induced in the first cell that became malignant. On the other hand, the cytological anomalies of the primary tumor may have originated after the malignant transformation had taken place. There is some evidence that the agents used in carcinogenic tests can remain present in the developing tumor. Cysts or "blisters" have been found in several primary tumors produced by the two isomers of NN-di-(2-chloropropyl)β-naphthylamine (CB 1075 and 1076). The contents of these cysts were found to contain the original component in measurable quantities. It is very likely that some mitotic abnormalities in the primary tumor were induced by these agents in this way. The validity of this case is supported by the fact that similar abnormalities have been observed in the cells of Walker carcinoma after a single intraperitoneal administration of the same aromatic mustard.

Conclusion

It has been shown that the alkylating agents produce a variety of cytological effects, and that among these the injuries to the chromosome mechanism of dividing cells are the most conspicuous. While there is evidence that the chromosome injuries of the radiomimetic types are directly induced by these agents, the excessive chromosome fragmentation and the breakdown of mitotic mechanism observed after the administration of several alkylating compounds may be the result of metabolic disturbances. It has been illustrated also that inhibition of growth in experimental tumors is not brought about by the radiomimetic injuries alone; other events such as suppression of mitosis, cell pyknosis in the resting stage, and stromal reaction also play a role. The selective action of Myleran on the granulocytes is further evidence for the fact that the ability of alkylating agents to produce radiomimetic injuries in cells is not necessarily the most important property in determining the effectiveness of these drugs in cancer therapy.

References

Auerbach, C., J. M. Robson & J. G. Carr. 1947. The chemical production of mutations. Science. **105**: 243–247.

Bergel, F. & J. A. Stock. 1954. Cyto-active amino-acid and peptide derivatives. Part I. Substituted phenylalanines. J. Chem. Soc. **2**: 2409–2417.

Dustin, P. 1947. Some new aspects of mitotic poisoning. Nature. **159**: 794.

Galton, D. A. G. 1953. Myleran in chronic myeloid leukaemia. Lancet. **264**: 208.

Gellhorn, A., A. Kells & M. Golino. 1955. Mammary adenocarcinoma 755, glioma 26 and Brown-Pearce carcinoma. Cancer Research. **3** (Suppl.): 38–43.

Haddow, A. & G. M. Timmis. 1951. Bifunctional sulphonic acid esters. Acta Unio Intern. contra Cancrum. **7**: 469–471.

Haddow, A. & G. M. Timmis. 1953. Myleran in chronic myeloid leukaemia. Lancet. **264**: 207.

Hendry, J. A., F. L. Rose & A. L. Walpole. 1951. Cytotoxic agents. I. Brit. J. Pharmacol. **6**: 201–234.

Koller, P. C. 1947. Experimental modification of nucleic acid systems in the cell. I. Nucleic acid. Symposia Soc. Exptl. Biol. : 270–290.

Koller, P. C. & A. Casarini. 1952. Comparison of cytological effects induced by X rays and nitrogen mustard. Brit. J. Cancer. **6**: 173–185.

Koller, P. C. 1953. Dicentric chromosomes in a rat tumour induced by an aromatic nitrogen mustard. Heredity. **6** (Suppl.): 181–196.

KOLLER, P. C. 1955. Mitotic poisoning by 4 aminostilbenes. J. Natl. Cancer Inst. **15**: 1237–1265.

KOLLER, P. C. & U. VERONESI. 1957. The effects of the nitrogen mustard derivative of phenylalanine amino acid on experimental tumours. Brit. J. Cancer. **11.** In press.

SUGIURA, K. 1955. Studies in a spectrum of mouse and rat tumours. Cancer Research. **3** (Suppl.): 18–27.

A NEW HYPOTHESIS FOR THE INTERPRETATION OF CHROMATID ABERRATIONS, AND ITS RELEVANCE TO THEORIES FOR THE MODE OF ACTION OF CHEMICAL AGENTS

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Introduction

In this paper I intend to draw attention to the implications of a new hypothesis (Revell, 1955) for the interpretation of a particular class of visible chromosome changes called *chromatid* changes. These aberrations are usually assumed to arise entirely from breaks induced in separate chromatids; for this reason they are sometimes called *post-split* changes.

In nearly all the work of testing the hypothesis which has been done so far, X rays have been used to induce the aberrations. On this occasion, however, the evidence to be given comes from a relatively small experiment with a diepoxide.

In experiments such as these, it must always be emphasized that although the chromosome aberrations are initiated in interphase, they can be scored only when the chromosomes next appear; that is, at the next mitosis. Thus, a chromosome development, which is of unknown complexity, occurs between the primary biological effect of treatment (whatever this primary effect may be) and the conversion of the aberration to an observable state. The hypothesis to be described is not concerned primarily with the manner in which the aberrations develop but simply with their structural interpretation at metaphase of mitosis, when their development is already completed. It will be pointed out that as a method of interpreting chromatid aberrations at metaphase the hypothesis yields internally consistent results. However, in spite of this limitation, the hypothesis does carry an important implication about the earlier development of the aberrations, and this will be explained at the end of the paper.

All the work described here has been done with root-tip cells of the broad bean (*Vicia faba*). The diepoxide used was di-(2,3-epoxypropyl)ether; the roots were placed in an aqueous solution of strength 2.5 × 10^{-4} *M* for 1 hour and then replaced in water for 20 hours before fixation.

The Exchange Hypothesis

The new hypothesis is based upon two directly observable facts.

The first fact is that, after chemical treatment (or irradiation), chromatid exchanges can be seen to have occurred between different chromosomes. Each exchange is between one of the two chromatids in one chromosome and one of the two chromatids in another chromosome; at a certain point they change partners (FIGURE 1, top left).

The second fact is that a proportion of such exchanges is *incomplete*

(Catcheside, Lea, and Thoday, 1946); that is to say, at the point of exchange two chromatid ends lie loose instead of being joined up in the exchanged relationship. Any exchange can be incomplete in one of two ways (FIGURE 1, top right).

Now, in any sample of treated cells seen at metaphase there are, in addition to the chromatid exchanges just described, other aberrations that look quite different. According to the hypothesis now proposed, all these other aberrations are also derived from chromatid exchanges but, in this case, between points so close together on the same chromosome that the exchange itself is no longer recognizable by the time the chromosome structure has contracted to the metaphase state.

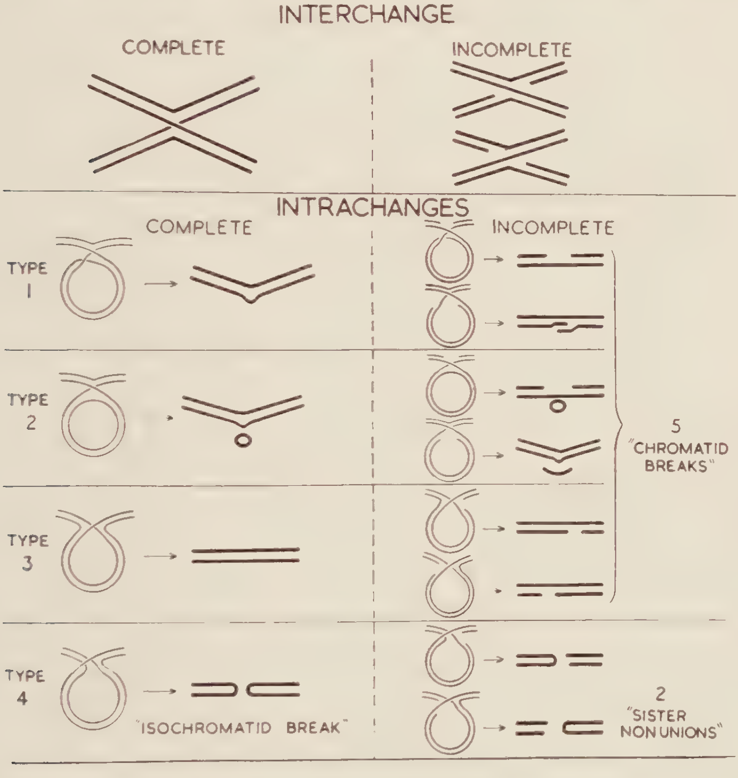

FIGURE 1. Diagram of the exchange hypothesis. Explanation in text.

In detail the hypothesis runs as follows. Consider a single chromosome with a small part coiled on itself in a single loop. If a chromatid exchange occurs at the point of association, then four results are possible, depending on which combination of chromatids is exchanged (FIGURE 1, bottom left, types 1 to 4). An internal exchange, or *intrachange*, of type 1 gives a small duplication-deletion (a short length of one chromatid is intercalated in the other). An intrachange of type 2 gives a small deletion from one chromatid in the form of a minute ring. Type 3 gives a small inversion in one chromatid. In type 4, sister chromatids are joined up with one another.

If it is assumed that each of these four types of *intra*change can be incomplete in two ways, just as the *inter*changes are observed to be, then eight more types of aberration are possible (FIGURE 1, bottom right):

A type-1 intrachange, incomplete either way, results in the appearance of a "chromatid break" in conjunction with the duplication deletion.

A type-2 intrachange, incomplete one way, results in a chromatid break at the point of deletion; incomplete the other way, it gives a "break" in the ring.

A type-3 intrachange, incomplete either way, gives a chromatid break in conjunction with the inversion.

A type-4 intrachange, incomplete either way, gives failure of joining between sister chromatids. This failure may be in either the centric fragment or the acentric one.

If we now return to the aberrations, other than obvious chromatid exchanges, that are actually observed at metaphase, it is apparent that this hypothesis can account for all the kinds commonly produced by low doses of a chemical mutagen (or ionizing radiation), on the assumption that all the intrachanges unravel by the time metaphase is reached:

Chromatid breaks (conventional terminology—ct) could arise from incomplete intrachanges of types 1, 2, or 3.

An isochromatid break (ct), with both fragments showing sister union (ct), could arise from a complete intrachange of type 4. The two possible incomplete forms of a type-4 intrachange could give isochromatid breaks with either proximal or distal sister nonunion (ct).

Occasional examples of apparent duplication-deletions are seen at metaphase, in which a flexure of the chromosome occurs at a certain point due to the fact that one chromatid is longer than the other. It is proposed that such aberrations are derived from complete type-1 intrachanges.

More frequently one sees examples of chromosomes with a bend similar to that just mentioned, but with a small minute (ct) close by. It is proposed that these arise from type-2 intrachanges.

On such a hypothesis it is to be expected that intrachanges of types 1, 2, and 3 will not be scored efficiently at metaphase in their complete forms because of the difficulty of seeing them in their unraveled state. On the other hand, if types 1, 2, and 3 are incomplete, then they will be scorable because, with one exception, they will appear at metaphase as chromatid breaks (ct). The exception is one of the incomplete type-2 intrachanges that gives a broken minute (ct) and not a chromatid break (ct). Intrachanges of type 4 (isochromatid breaks) will be scored efficiently both in their complete and incomplete states.

A Procedure for Testing the Hypothesis

In order to test the hypothesis it has been necessary to make two assumptions, either of which might prove to be unsound, but both of which seemed reasonable at the outset:

(1) Let the four types of intrachange occur with equal frequencies and (2) let there be an equal likelihood that any chromatid exchange is incomplete (this is to apply to both *inter*changes and *intra*changes).

On this basis it is possible to make certain simple predictions (FIGURE 1). In any given treated cell sample it should be possible to make three different estimates of the likelihood that chromatid exchanges are incomplete, and these three estimates should be the same. This may be done in the following way:

First, an estimate may be obtained of the proportion of interchanges (chromatid exchanges between different chromosomes) that are incomplete (p_1). Second, an estimate may be obtained of the proportion of isochromatid breaks that show either proximal or distal sister nonunion or, in other words, the proportion of type-4 intrachanges that are incomplete (p_2). The two estimates of incompleteness (p_1 and p_2) should agree: in fact, such an agreement has been observed by Catcheside, Lea, and Thoday (1946) and by Conger (1955) in irradiated material. Third, it should be found that for every 2 sister nonunions observed there is a mean of 5 chromatid breaks; in other words, the total number of incomplete intrachanges of types 1, 2, and 3 that yield chromatid breaks should be equal to 2.5(incomplete type-4 intrachanges).* It is convenient to derive from the observed number of chromatid breaks an estimate of the proportion of intrachanges of types 1, 2, and 3 that are incomplete (p_3) for comparison with the other two estimates (p_1 and p_2). This is done by dividing the number of chromatid breaks by a number equal to 2.5(*total* isochromatid breaks): it is necessary to deduce this latter number because the complete intrachanges of types 1, 2, and 3 cannot be scored efficiently; thus their real total cannot be observed.

If the two assumptions are correct, namely, that all four types of intrachange are equally frequent, and that the likelihood of incompleteness is the same for all exchanges, then the estimates p_1, p_2, and p_3 should not differ significantly.

It must also be expected that the actual number of minute rings should equal the number of isochromatid breaks. In practice, most likely it will prove impossible to demonstrate this equality because of the relative difficulty of scoring minutes; but it is necessary to predict that the number of minutes observed should not exceed the number of isochromatid breaks.

Experiments

As already mentioned, nearly all the analyses that so far have been made to test the hypothesis given above have been on irradiated cell populations. These results will be published in detail elsewhere, but it is relevant to say here that they show good agreement with the predictions already given.

The estimates of p that have been obtained indicate an incidence of incompleteness varying between about 6 and 10 per cent following doses of the order of 50 r of X rays. Within each experiment the three different estimates of p have not differed significantly. Also, the numbers of minutes observed have been between one half and three quarters of the respective numbers of isochromatid breaks, and these, having regard to the difficulty of detecting minutes, are considered quite acceptable figures.

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* It should be remembered that there are only 5 and not 6 chromatid breaks for every 2 sister nonunions because the broken ring (type 2) does not appear as a chromatid break.

The data given in this paper were obtained from a relatively small analysis of 1000 cells that had been treated with di-(2,3-epoxypropyl)ether. The results are shown in FIGURE 2.

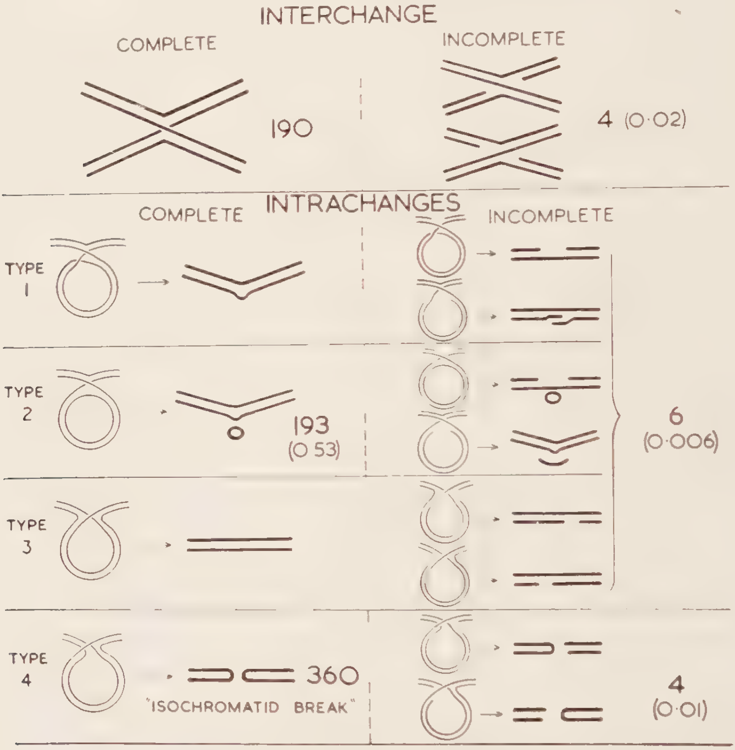

FIGURE 2. Data from an experiment, designed to test the exchange hypothesis, in which the aberrations were induced by 2.5×10^{-4} *M* di-(2,3-epoxypropyl)ether. Explanation in text.

Of the 1000 cells examined, 549 carried chromatid aberrations. There were 194 chromatid *inter*changes; of these, 4 were incomplete ($p_1 = 0.02$). There were 364 isochromatid breaks (≡ type-4 intrachanges); of these, 4 showed either proximal or distal sister nonunion ($p_2 = 0.01$). There were 6 chromatid breaks observed ($p_3 = 0.006$). Finally, there were 193 minutes scored, compared with 364 isochromatid breaks—a proportion of 0.53.

These figures are, of course, very small and, taken by themselves, they could not be regarded as at all convincing evidence for the hypothesis proposed. However, they gain significance by comparison with the X-ray results already mentioned: from a consideration of both sets of results it appears reasonable to conclude that the proportions of incomplete exchanges observed after diepoxide treatment are extremely small. Clearly, it would be necessary to do a much larger analysis of diepoxide-treated material to obtain a more accurate observation.

Discussion

As was pointed out in the *Introduction*, the hypothesis discussed in this paper is for the structural interpretation of chromatid aberrations as seen at metaphase. Although it is proposed that all these changes have arisen from chromatid exchanges, there are no specific proposals as to how each exchange develops. However, it will perhaps be apparent already that the hypothesis does lead to a certain general conclusion about the nature of the exchange. This conclusion is derived as follows.

It must first be emphasized that all the aberrations, including those conventionally interpreted as chromatid and isochromatid breaks, are considered to rise from chromatid exchanges. Hence the "breaks," according to this view, are no longer simply surviving examples of the primary units of biological change, as they are usually interpreted to be. Each chromatid exchange is a recombination between either one or the other chromatid of one chromosome *and* either one or the other chromatid of another chromosome. Such a chromatid exchange could not be the only product of a situation in which chromatids, or precursory chromosomes, were broken at random and reunited at random.

Therefore, if the new hypothesis provides a correct interpretation of chromatid aberrations at metaphase, it must also be concluded that the pairs of chromosome loci involved have a *capacity* to undergo only chromatid exchange during their previous development. This competence to exchange is activated by the chemical agent (or the radiation). This does not appear to be at all an unacceptable conclusion, especially since it is known that such exchanges can occur at meiosis under physiological control.

It is this implication of the new hypothesis that I want to stress in these pages, because it is very difficult to see how the primary response, at the biological level, in any such exchange process can be one of breakage of the chromosome.

Acknowledgments

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References

Catcheside, D. G., D. E. Lea & J. M. Thoday. 1946. Types of chromosome structural change induced by the irradiation of *Tradescantia* microspores. J. Genet. **47**: 113–136.

Conger, A. D. 1955. J. Cellular Comp. Physiol. **45** (Suppl. 2): 309–312.

Revell, S. H. 1955. A new hypothesis for "chromatid" changes. Proc. Radiobiol. Symposium (1954, Liège). : 243–253. Butterworth. London, England.

DISCUSSION

HOWARD E. SKIPPER [*Kettering-Meyer Laboratory (affiliated with Sloan-Kettering Institute for Cancer Research, New York, N. Y.), Southern Research Institute, Birmingham, Ala.*]: The very interesting data presented by Lois Murphy indicate that the rapidly growing rat fetus is more sensitive to alkylating agents than is the maternal host, considered as a living unit; also that at one half to two thirds of an LD_{50} dose such alkylating agents are monster-producing or mutagenic for the mammalian embryo.

The fine paper contributed by P. C. Koller emphasizes the fact that alkylating agents "produce a variety of cytological effects, and that among them the injuries to the chromosome mechanisms of dividing cells are the most conspicuous." Koller has raised the question as to whether the radiationlike effects alone are responsible for the tumor-inhibiting action of the alkylating agents or whether other biological events such as suppression of mitosis, cell pyknosis in the resting stage, or stromal reaction also play a role. He suggested that some of these later effects may be the result of metabolic disturbances.

Revell has presented an interesting hypothesis for interpretation of chromatid aberrations induced by chemical agents and ionizing radiation.

It would be presumptuous and fruitless for me to attempt to belabor the conclusions drawn by these three investigators within the space at my disposal. Comments on these fine papers appear elsewhere in these pages, and further discussion of them doubtless will continue for months to come.

It appears to me that Murphy, Koller, and Revell might agree that alkylating agents are "mutagenic" to mammalian cells; are "damaging" to rapidly growing cells; cause "injury" to the chromosome mechanism of dividing cells; and that these biological effects might be the result of chemical reaction between alkylating agents and molecular constituents of cells which in turn causes dire metabolic "disturbances."

I propose here to present certain data on a molecular level that possibly may be related to the cytological and embryological observations that have been described.

The difficulty associated with any attempt to prove the primary site of the biological action of alkylating agents on a biochemical level may be found in their nonspecificity with regard to alkylation. However, the poor chemist cannot help but be influenced and titivated when he hears again and again the following terms and statements made regarding the biologic action of alkylating agents: (1) that these agents have induced gene mutation at minute doses; (2) that their lethal effects are nucleotoxic; (3) that they have direct effects on chromosomes; (4) that the dynamic centers of the chromosomes are particularly sensitive to them; (5) that they cause breakage of chromatids at high frequency; (6) that they account for the stickiness of chromosomes; (7) that the coiling mechanism of nucleoprotein may be affected by them; (8) that they account for chromosome breaks and bridges; (9) that they inhibit mitotic division at levels that fail to affect respiration and fermentation; (10) that the biologic units most sensitive to alkylating agents appear to be viruses and the transforming factor deoxyribonucleic acid (DNA); and (11) that certain alkylating agents are mutagenic, carcinogenic, and tumor-inhibiting in mammals.

Such comments cause a chemist who is interested in nucleic acid metabolism and cancer to wonder if all these suggestive biological observations are not the result of varying degrees of reaction between alkylating agents and gene chemicals or cellular polynucleotides. Such inquiry is apt to lead him to carry out experiments to see if such an obvious explanation is at least feasible.

In 1951 my colleagues and I observed that nitrogen mustard, when injected at 4 mg. kg., slightly inhibited formate incorporation into the polynucleotides of mouse tissues while failing to inhibit formate incorporation into other tissue components.[1]

Goldthwait[2] found that nitrogen mustard (4 mg. kg.) decreased formate or adenine incorporation into DNA of the rat intestine by about 75 per cent, but failed to affect ribonucleic acid (RNA) biosynthesis. The dosage levels used in these experiments would provide, assuming equal distribution of mustard in the animal, tissue concentrations of about 2.5×10^{-5}.

Such knowledge as this led my associate, Glynn P. Wheeler,[3, 4] to begin studies on the alkylation of polynucleotide moieties by mustards both *in vitro* and *in vivo*. Using C^{14}-labeled nitrogen mustard and S^{35}-sulfur mustard, Wheeler demonstrated that mustards would indeed interact *in vitro* with a variety of purines and pyrimidines, forming stable reaction products. No substitution on the purine ring is required for such a reaction because unsubstituted purine will form a single reaction product with mustard. Nor is a hydrogen atom directly associated with a ring nitrogen atom necessary for the mustard-purine reaction, since 1,3,7-trimethylxanthine can be alkylated by mustard.

The next step in Wheeler's approach was to show that nucleic acid purines alkylated *in vitro* with methyl-labeled nitrogen mustard were of sufficient stability to withstand the chemical procedures employed in nucleic acid purine isolations; that is, that the labeled methyl group would appear in the finally precipitated silver purines, and that such alkylated purines were precipitable with silver.

Studies were then carried out in which methyl-labeled nitrogen mustard was injected into mice at a level of 4.8 mg. kg. ($2\mu c.$ mouse) and combined tissue, sodium nucleates, DNA, and RNA purines isolated six hours later. For comparative purposes, carefully hydrolyzed labeled nitrogen mustard was injected in other mice at similar levels and the tissues were fractionated for activity assays.[5]

Typical results of such preliminary studies are summarized in TABLE 1. These results might be interpreted to suggest that nitrogen mustard does indeed alkylate polynucleotide purines of mammalian cells *in vivo* and that further work of this sort is worthwhile.

Other experimental results obtained by Wheeler have indicated that nitrogen mustard at concentrations of 10^{-3} in metabolizing cell suspensions inhibit purine nucleotide formation *de novo* and the formation of labeled nucleotides from labeled adenine. This work is being continued. Efforts are being made to determine the lowest level of nitrogen mustard that will affect nucleotide metabolism.

In summary, I hope that Wheeler and other chemists will be encouraged to study in living cells the degree and specificity of alkylation of cellular constituents; perhaps, particularly, polynucleotides and precursors of them in view of what Murphy, Koller, and Revell have described for us. Perhaps with such effort the labors of the chemist and the biologist, in time, will cohere.

TABLE 1

Fraction	Specific activities (μc./mole of carbon)		
	Following addition of hydrolyzed HN2-$C^{14}H_3$ to a "cold" tissue mince	Following injection of hydrolyzed HN2-$C^{14}H_3$ into mice	Following injection of HN2-$C^{14}H_3$ into mice
Tissue aliquot	5	—	13
Combined nucleic acids	0.05	0.32	15
DNA	0	0.16	8
RNA purines	<0.02	2.3	57

References

1. SKIPPER, H. E., J. H. MITCHELL, JR., L. L. BENNETT, JR., M. A. NEWTON, L. SIMPSON & M. EIDSON. 1951. Cancer Research. **11**: 145-49.
2. GOLDTHWAIT, D. A. 1952. Proc. Soc. Exptl. Biol. Med. **80**: 503–505.
3. WHEELER, G. P., J. S. MORROW & H. E. SKIPPER. 1955. Arch. Biochem. Biophys. **57**: 125.
4. WHEELER, G. P., J. S MORROW & H. E. SKIPPER 1955. Arch. Biochem. Biophys. **57**: 124.
5. WHEELER, G. P. Arch. Biochem. Biophys. In press.

PHARMACOLOGICAL AND PATHOLOGICAL EFFECTS OF ALKYLATING AGENTS*

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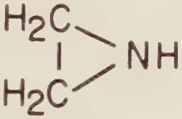
Monofunctional Agents

The alkylating agents are characterized by a high order of selective action against certain proliferative tissues: hematopoietic cells in bone marrow and lymphoid organs, the intestinal mucosa, and some neoplastic tissues. They are characterized further by the observation that, in any closely related series of monofunctional and polyfunctional derivatives, those that are more damaging are usually the ones in which reactive moieties are present at least in duplicate.[1] While certain monofunctional derivatives actually damage susceptible proliferative tissues, usually such effects occur with doses that cause other kinds of cytotoxicity or acute pharmacological changes or both.



ETHYLENIMINE

FIGURE 1

An early and classic example of such a change with a monofunctional derivative was described in 1896 by Gabriel and von Hirsch.[2] Their report was concerned in part with the toxic effects of ethylenimine (FIGURE 1) and α-methylethylenimine (then considered to be vinylamine and isoallylamine). These substances had been synthesized by Gabriel and his colleagues and supplied to Ehrlich for physiological testing. Ehrlich found what may well represent one of the first experimental observations of selective systemic damage induced by alkylating agents. Lethal doses produced localized damage of the renal papilla; the fully developed lesion resulted in complete papillary necrosis.[2, 3a] (Examples of this are seen in FIGURES *2* and *3*.) This lesion was studied by pathologists for a number of years following Ehrlich's initial observations, but only considerably later did it become evident that the renal toxicity was accompanied by bone marrow depression.[3b] Interestingly, the clinically useful polyethylenimines such as triethylene melamine or the triethylene phosphoramides have not caused nephrotoxicity, despite the presence of the ethylenimine moiety.

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Another informative series of monofunctional derivatives are the close analogues of bis(β-chloroethyl)methylamine (HN2) shown in TABLE 1. These mono-β-chloroethylamines are 50 to 100 times less potent (that is, less toxic) than HN2.[1, 5] When given in lethal doses, they produce in mice a neurological syndrome similar to that seen in the mutant strains known as "waltzers" or "shakers."[5] The syndrome is associated with lesions in the cerebellum, medulla, and upper cord. Supralethal doses of HN2 and related bis(β-chloroethyl)amines cause neurological abnormalities and hyperactivity in mice resembling in part the induced "waltzing" syndrome. At such doses, however, mice do not survive long enough fully to develop the selective neuropathy. Conversely, the mono-β-chloroethylamines are not entirely ineffective against proliferative tissues. For instance, β-chloroethyldi-

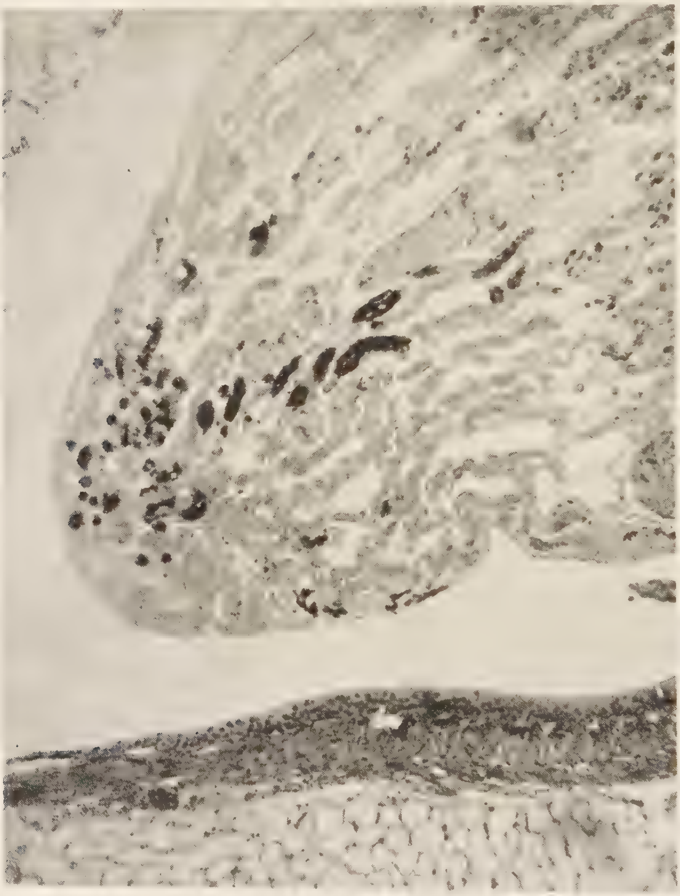

FIGURE 2. Diffuse necrosis of the terminal portion of renal papilla (rat given a single 12 mg. kg. injection of ethylenimine and sacrificed 12 days later). At the extreme right a few partially intact tubules can be seen. The dark foci are collections of red blood cells.

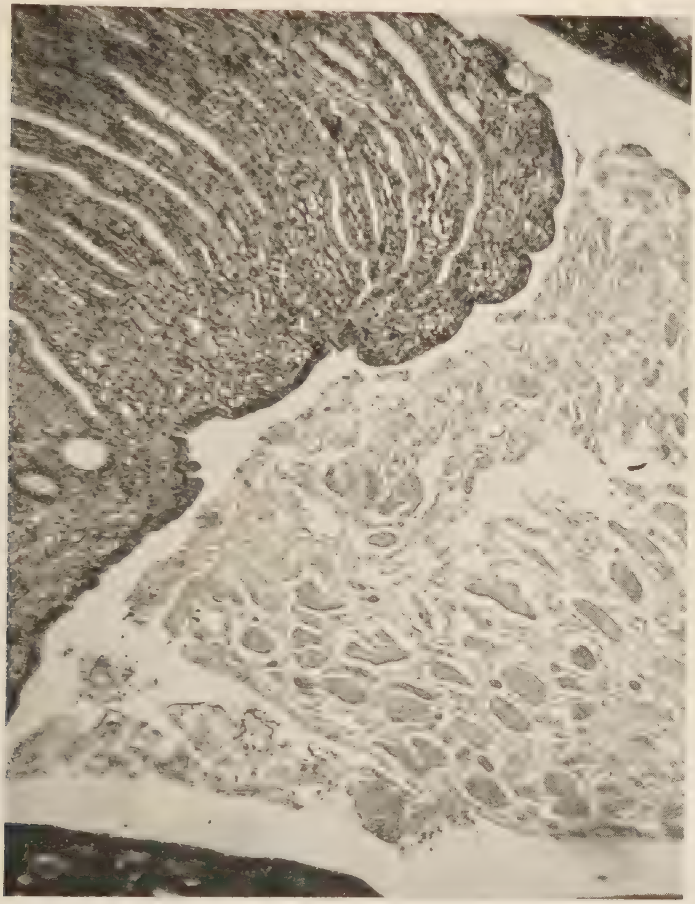

FIGURE 3. Remains of necrotic papilla lying free in the renal pelvis after separation from parenchyma (rat given a single 6 mg./kg. injection of ethylenimine and sacrificed $2\frac{1}{2}$ months later). Epithelium has regenerated and now covers viable proximal portion.

TABLE 1

LD_{50} IN MICE, SINGLE DOSES, I.P.

	mM/kg.
Bis(β-chloroethyl)methylamine·HCl	0.02[4]
β-Chloroethylethylmethylamine·HCl	1.30[5]
β-Chloroethyldiethylamine·HCl	0.41[5]
β-Chloroethyldimethylamine·HCl	1.95[5]

methylamine (TABLE 1) has been found to be an effective inhibitor of experimental tumors, but such inhibition is obtained only with doses a thousand-fold greater than those of HN2.[6]

Monofunctional agents do not elicit only untoward or useless effects. Drugs such as Dibenamine and Dibenzyline (FIGURE 4) have well-established utility as potent adrenergic blocking agents.[7] Their prolonged effects have been attributed, at least in part, to irreversible alkylation of adrenergic receptor sites. Certain Dibenamine-like substances, such as α-naphthylmethyl-ethyl-β-chloroethylamine, have antihistaminic properties in addition to their adrenolytic effects. These compounds occasionally cause unwanted central stimulation manifested by restlessness, nausea, and disturbances of time perception. They do not, however, produce pathological changes in proliferative or other tissues.

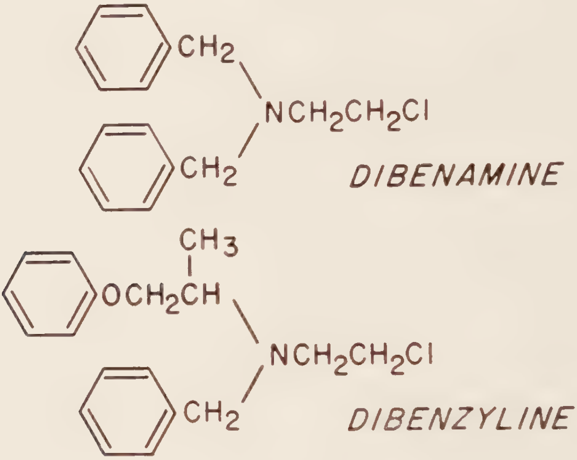

FIGURE 4

Polyfunctional Agents

Pharmacological effects. The polyfunctional alkylating agents vary widely in potency (TABLE 2) with respect to lethality, bone marrow depression, and inhibition of neoplastic tissue.[1, 8] These quantitative variations are well established and are readily apparent when comparisons are made within series containing the same reactive moiety or between series containing different reactive groups: β-chloroethyl, ethylenimine, and methanesulfonoxy (TABLE 2). It is important to ascertain whether such quantitative variations in over all potency are truly associated with modifications of the proportion between doses causing harmful effects and those eliciting useful therapeutic responses. If real, such modifications might distinguish polyfunctional alkylating agents qualitatively with respect to therapeutic efficacy.

It is appropriate to begin discussion of qualitative differences with reference to the complex pharmacological effects of HN2. Characteristically, two

TABLE 2

LD_{50} IN RATS, SINGLE DOSES, I.P.

	mg./kg.	mM/kg.
HN2·HCl	1.8	0.009^{4}
Chlorambucil	23	0.08
Triethylene melamine	1.0	0.005^{4}
Triethylene thiophosphoramide	8.4	0.04
Myleran	18	0.07
1,9-Dimesylnonane	250	0.8

readily separable types of toxic response may be produced by different dose ranges (FIGURE 5).[8] On the one hand, in the range of the median lethal dose (6.2 to 12.5 mg./kg.), death is delayed and is associated with the marked changes in proliferative tissues to be discussed later. On the other hand, supralethal doses (25 to 50 mg./kg.) elicit acute, fulminating fatal changes. In this high dose range, paralytic and cholinergic effects are seen that are probably due to the formation *in vivo* of cyclic quaternary ethylenimonium derivatives. The parasympathomimetic actions of HN2 seemingly are related to the cationic nature of the quaternary imonium transformation products, which closely resemble acetylcholine in structure.[9] Analogues of HN2, in which the methyl group is replaced by ethyl or β-chloroethyl, are also known to transform into quaternary ethylenimoniums; these analogues are also cholinergic. Similarly, the cholinergic properties of sulfur mustard may be associated with its transformation *in vivo* into the cyclic tertiary ethylenesulfonium state.[1] The cholinergic and paralytic properties of the aliphatic nitrogen and sulfur mustards are not shared by the aromatic nitrogen mustards, polyethylenimines, or methanesulfonoxy derivatives.

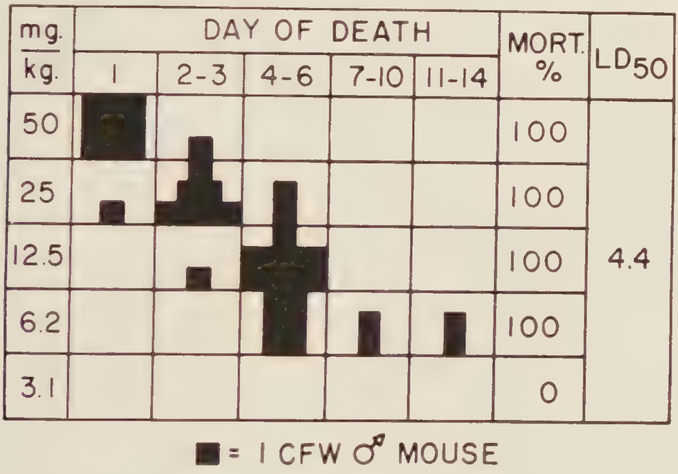

FIGURE 5. Toxicity of single I.P. doses of HN2·HCl (dissolved in isotonic saline).

This is consistent with the fact that the last-mentioned compounds do not form cationic onium derivatives.[10]

Central nervous system stimulation with convulsions is another acute pharmacological effect of bis(β-chloroethyl) derivatives. Convulsions are not prominent in rodents given HN2, but they do occur in cats. The close analogues of HN2, with ethyl or β-chloroethyl replacing the methyl group, and sulfur mustard are even more potent convulsants. This effect is peculiar to the untransformed, parent compounds; convulsions do not occur after the administration of the respective cyclic ethylenimonium derivatives.[1, 9] Perhaps the central nervous system sites are more accessible to the lipid-soluble, unchanged bis(β-chloroethyl)amines.

Convulsant properties are not limited to the aliphatic nitrogen and sulfur mustards. The aromatic nitrogen mustard, chlorambucil, *p*-bis(β-chloroethyl)aminophenylbutyric acid, causes convulsions in mice and rats and kills them within a few hours after the intraperitoneal administration of median lethal doses.[8] Dimethanesulfonoxy derivatives, such as Myleran and its nonane analogue, are also acutely convulsant in mice and rats. In this instance the intensity of the seizures may be somewhat attenuated by the simultaneous appearance of depression.[8] As will be seen below, the acute actions of Myleran are elicited in mice after the intraperitoneal administration of median lethal doses. In rats the same effects appear only when considerably larger amounts are given. Finally, in contradistinction to the other classes of alkylating agents, the polyethylenimines appear to be free of convulsant activity.[4]

TABLE 3

MORTALITY AND CONVULSIONS IN CATS FOLLOWING INTRAVENOUS ADMINISTRATION OF SK136, 1,3-DI[BIS(2-CHLOROETHYL)AMINO]PROPANE DIHYDROCHLORIDE

Dose mM/kg.	Mortality	Day of death	Convulsions
0.02	1/1	1	1/1
0.01	4/4	1, 1, 1, 1	4/4
0.005	6/7	1, 2, 3, 7, 7, 8	6/7
0.0025	1/4	8	0/4
0.002	0/1		0/1

It appears that the central stimulation produced by bis(β-chloroethyl)amines is not a mere laboratory curiosity. It may well be related to the toxic psychoses seen in patients given certain derivatives. An early example of this was discovered during the clinical trials of two closely related substances: 1,3-di[bis(β-chloroethyl)amino]propane and its 2-chloro derivative (TABLE 3). In both, convulsions were observed in cats at doses equivalent to those causing delayed intoxication.[11] The first caused dizziness in patients, the second, toxic psychoses.[12]

The nauseant and emetic effects of HN2 and other bis(β-chloroethyl)amines are possibly also related to central stimulation.[13] This effect, too, is of considerable practical importance in the clinic. The polyethylenimines and dimethanesulfonoxy derivatives are apparently superior to HN2 in this respect, since they are less nauseant in patients.

These pharmacological effects, although incomplete, do permit discernible distinctions of different classes of polyfunctional alkylating agents. These comparisons may be summarized as follows:

(1) The aliphatic bis(β-chloroethyl) amines and sulfides are, in supralethal and near lethal doses, complex in action: cholinergic, paralytic, and central nervous stimulatory effects are common.

(2) The aromatic nitrogen mustards and the methanesulfonoxy compounds are not known to have parasympathomimetic properties. They can cause convulsions.

(3) The polyethylenimines are relatively free of all acute pharmacological properties.

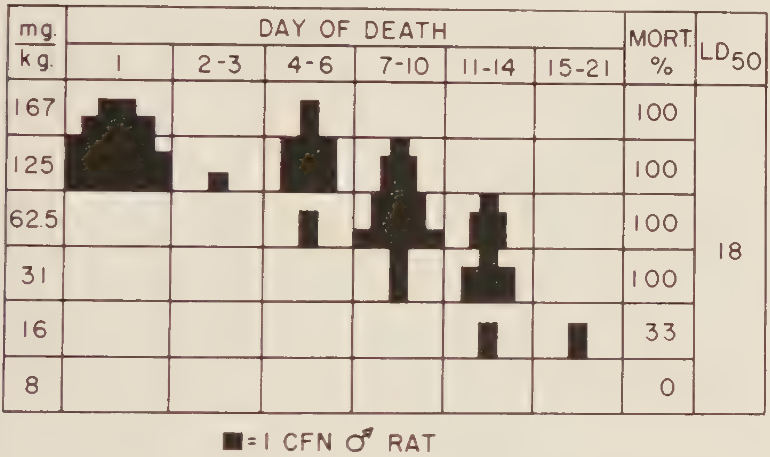

FIGURE 6. Toxicity of single I.P. doses of Myleran (suspended in peanut oil).

It should be emphasized that these distinctions are based largely on studies of response to supralethal or median lethal doses. In the practical sense, the distinctiveness of individual polyfunctional compounds may be lost when doses are at sublethal and chemotherapeutic levels. The acute studies may possibly have served to clarify variations in emetic activity.

Pathological effects in animals. Previous studies of pathological changes produced by certain of the polyfunctional alkylating agents have been performed for the most part in animals given compounds such as HN2 in single lethal doses at or near the LD$_{50}$. The properties of HN2 are convenient for this purpose, since death is sufficiently delayed to permit pathological changes to develop fully before death or sacrifice of the animal. The observed alterations are sufficiently severe to account for the lethal outcome. The properties of Myleran in the rat are also ideal for the same purpose (FIGURE 6).[8] In the mouse, however, single doses of Myleran at or near the LD$_{50}$ killed a number of the animals within the first day; others died after a prolonged course of intoxication, and a significant number survived (FIGURE 7).[8] Thus, in the mouse, pathological studies might be somewhat complicated by the rapid death of the more sensitive animals.

A more serious problem might attend studies of compounds such as chlorambucil which, with single doses, kills mice and rats within a matter of hours. In this instance the convulsant dose may be equal to, possibly even less than, the dose causing significant pathological changes. Accordingly, it may be difficult to obtain a significant comparison of differences between the lesions produced by such a compound and those caused by agents such as HN2 or Myleran.

The experiments to be described were designed primarily for the purpose of obtaining illustrative pathological material. Two alkylating agents, HN2 and Myleran, were used. Young male Wistar rats received single intraperitoneal injections of HN2, either 2 mg./kg. or 6 mg./kg., or Myleran, 62.5 mg./kg. A fourth group of animals served as controls. The smaller dose of HN2 was approximately the LD_{50}. The 6 mg./kg. dose of HN2 and the Myleran dose were approximately three times the LD_{50}. Work at the Chester Beatty Research Institute in London has indicated that Myleran at the LD_{50} level causes principally bone marrow damage.[14, 15] It was hoped that by the use of supralethal doses the lymphoid and intestinal damage of HN2 might be duplicated. Animals receiving HN2 were sacrificed at 1, 2, and 3 days; Myleran animals at 2, 4, and 7 days. With the higher dose of HN2, deaths occurred after the third day; with Myleran, after 7 days. The pathological changes seen following HN2 were essentially similar to those previously described by Graef *et al.*,[16] Karnofsky *et al.*,[17] and Kindred.[18]

Lymphoid tissues. In both groups of animals receiving HN2, complete involution of the thymus was seen at the third day. This was characterized by a decrease of lymphocytic elements, reduction in size of the organ, and loss of corticomedullary demarcation. The same changes occurred with Myleran, but only at the end of 7 days. Karyorrhexis was prominent in the thymus of the rats sacrificed at 24 hours after the higher dose of HN2; at this

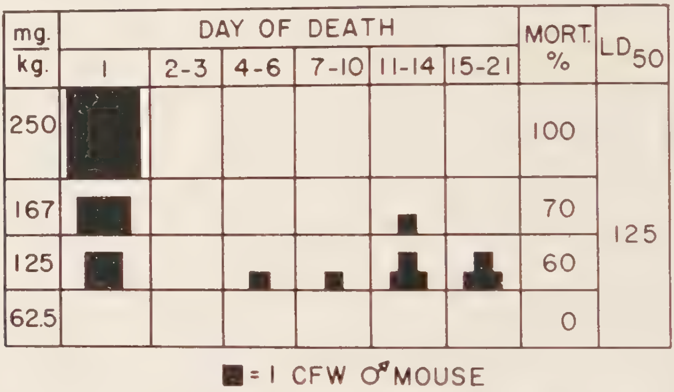

FIGURE 7. Toxicity of single I.P. doses of Myleran (suspended in peanut oil).

time it was only minimal in those given the dose of 2 mg. kg. The lesion was transient; animals sacrificed at 2 and 3 days did not show the nuclear fragmentation. Significant karyorrhexis was not seen in the Myleran-treated rats but, in view of the rapidity with which nuclear debris is removed, it cannot be concluded that the agent did not elicit this change.

Cervical and mesenteric lymph nodes were examined also. Certain changes occurred that were common to all treated animals. These were a decrease in size, a loss or blurring of the follicular pattern, and a disappearance of germinal centers. In addition to these alterations the animals receiving the higher dose of HN2 showed marked karyorrhexis at 24 hours and a striking diminution in the number of lymphocytes by the end of 3 days. The loss of lymphocytes appeared to be especially prominent in the mesenteric group of nodes. Lymphocyte depletion in the earlier stages occurred mainly in the cortex, where only shadowy outlines of the follicles remained (FIGURE 8). Later, the follicular pattern was lost, and with this there was a loss of demarcation of the cortex and medulla. In animals treated with Myleran or the smaller dose of HN2, although decreases in the size of the lymph nodes were obvious, significant amounts of lymphoid tissue nevertheless persisted (FIGURE 9). The contraction that occurred apparently took place in a symmetrical and diffuse fashion without the intervening changes described following the higher dose of HN2.

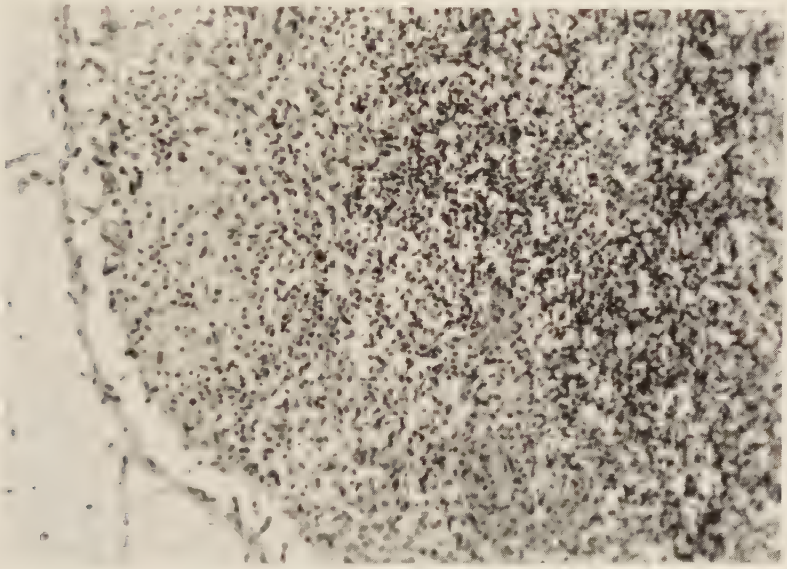

FIGURE 8. Lymph node (rat given a single injection of 6 mg./kg. of HN2 and sacrificed 1 day later). Marked decrease of lymphocytic elements chiefly in the cortex. The outline of the follicle can be seen at left.

In regard to changes in the spleen, fewer differences were observed among the three groups of animals. Thus, the reduction in the size of this organ

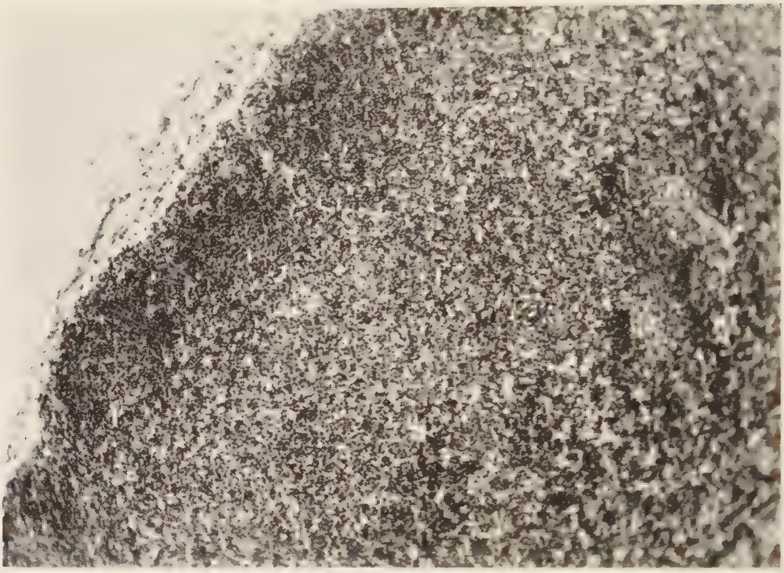

FIGURE 9. Lymph node. Moderate amount of lymphoid tissue still present 3 days after one 2 mg./kg. dose of HN2. Myleran animals showed very similar changes after the fourth day.

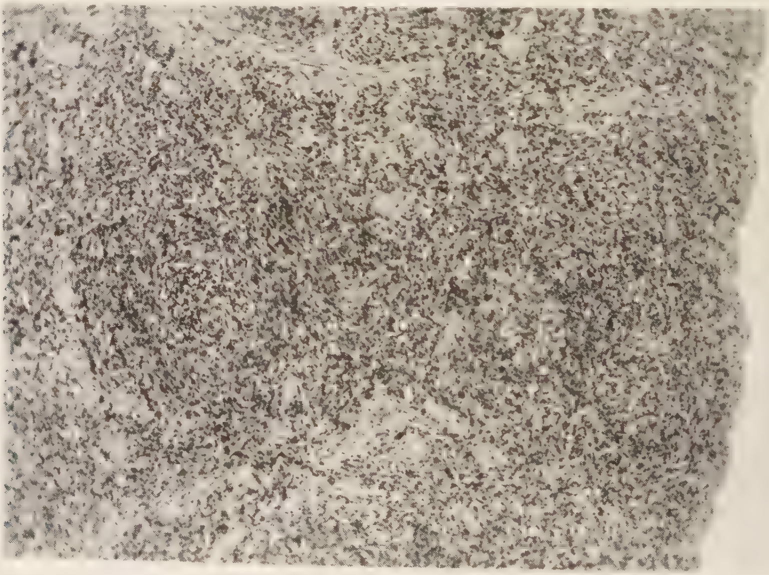

FIGURE 10. Spleen (rat given a single 62.5 mg./kg. injection of Myleran and sacrificed 7 days later). Only a few lymphocytes persist around the follicular vessels. The remainder of nucleated elements of pulp are small spindle cells. Rats given single 6 mg./kg. injections of HN2 and sacrificed on the third day showed virtually the same changes as seen here.

in animals sacrificed at 3 days after the higher dose of HN2 was similar to that seen at 7 days in animals treated with Myleran. The microscopic changes in both groups were identical. The follicles were either markedly reduced in size or were absent. The pulp was diffusely contracted, and the hematopoietic elements were replaced by small spindle cells (FIGURE 10). At the lower dose of HN2 the follicles were only moderately reduced, and the pulp was congested and relatively free of nucleated hematopoietic elements.

Sternal marrow. Decalcified sections of sternal marrow of animals receiving the 6 mg. kg. dose of HN2 revealed nearly 100 per cent depletion of nucleated elements at the end of 48 hours. A similar degree of aplasia was observed at the end of 4 days in the Myleran-treated group. The 2 mg. kg. dose of HN2 did not produce the nearly absolute depletion caused by either Myleran or the higher dose of HN2. At most, 80 to 90 per cent of the nucleated elements were absent at the end of 2 days; at 3 days the depletion varied from 60 to 85 per cent.

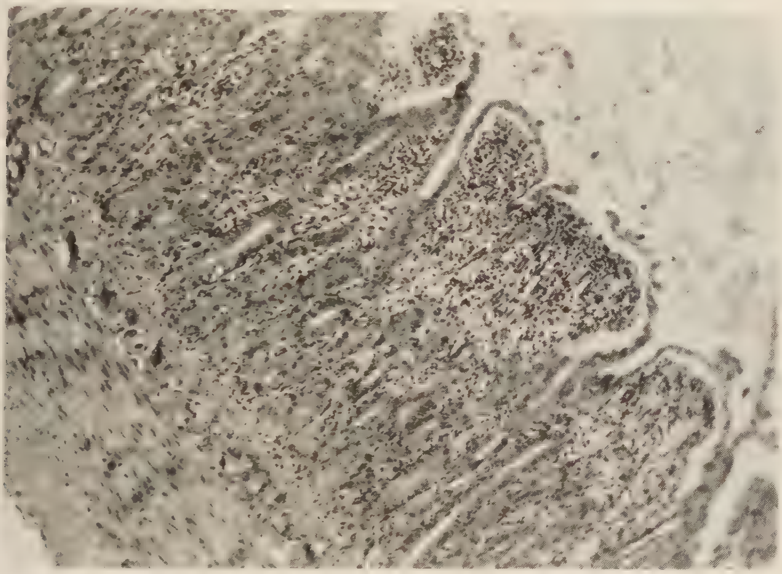

FIGURE 11. Small intestine (single injection of 62.5 mg. kg. of Myleran and sacrificed on fourth day). Irregularity and slight dilation of mucosal glands, loss of nuclear polarity and irregular nuclear swelling. Similar changes were observed in HN2 animals at 2 days.

Small intestines. One day after a 2 mg. kg. dose of HN2, nuclear swelling and loss of polarity occurred in the deeper portions of the mucosa. This was accompanied by some karyorrhexis. At the higher dose these changes were more severe. Two days after Myleran the lesion was like that seen after 2 mg. of HN2, but without karyorrhexis. All the HN2 animals at 2 days and the Myleran animals at 4 days showed marked atypia, loss of nuclear polarity, and moderate atrophy (FIGURE 11). At 3 days these changes were

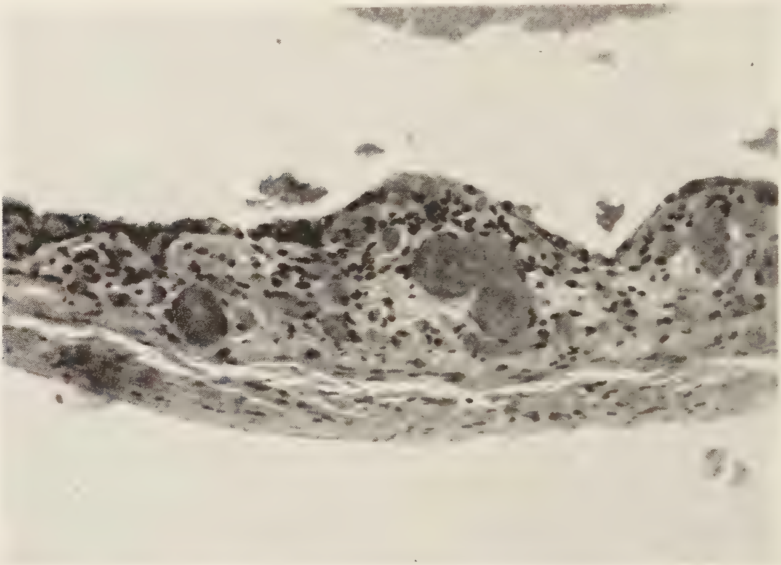

FIGURE 12. Large intestines. Example of severe colonic atrophy seen with a single 6 mg./kg. dose of HN2 at the end of 3 days. This lesion was seen also in some Myleran animals at 7 days.

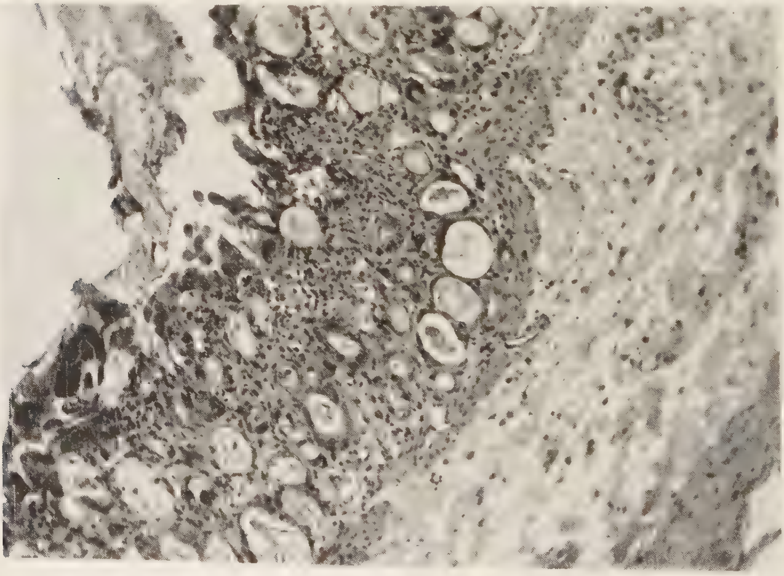

FIGURE 13. Large intestines. Atrophy, glandular ectasia, and submucosal edema at the end of 7 days after a single 62.5 mg./kg. injection of Myleran.

accentuated in the HN2 animals and, in addition, there were superficial erosions. However, at the end of 7 days the Myleran group showed little or no change in this segment of the intestines.

Large intestines. The smaller dose of HN2 caused alterations that were similar to, but less marked than those seen in the small intestines. At the end of 3 days no erosions were observed and the atrophy was less pronounced. The higher dose of HN2, on the other hand, resulted in severe atrophy by the end of 3 days. In 2 animals the wall was thinned to the extent that perforation appeared imminent (FIGURE 12). In the case of Myleran, the colon appeared normal at 2 days; slight alterations were evident at 4 days; and severe changes developed by 7 days (FIGURE 13).

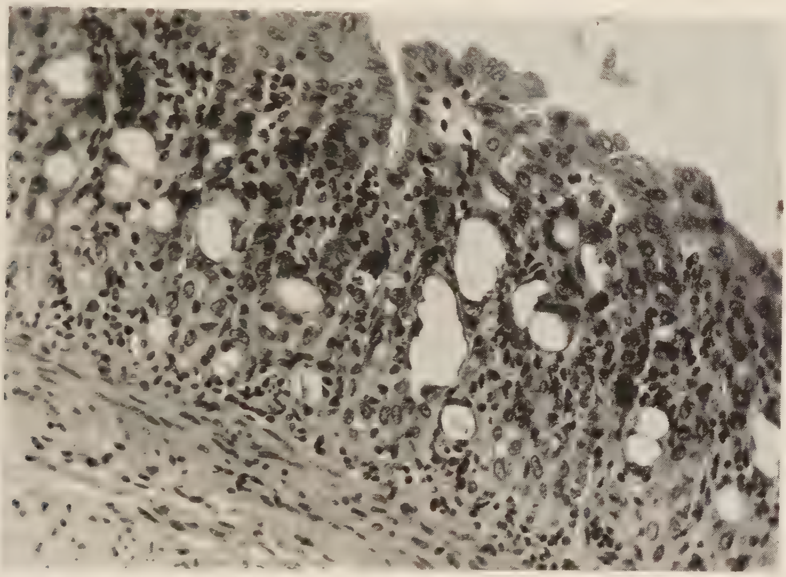

FIGURE 14. Stomach. Antral portion, showing glandular ectasia and nuclear irregularity 7 days after a single Myleran injection of 62.5 mg./kg.

Stomach. Alterations were found in the antrum of Myleran animals at the end of 7 days. These consisted of slight atrophy, glandular ectasia, and loss of nuclear polarity (FIGURE 14). In one animal similar effects were present in the fundus; in another there were hemorrhages in the fundus. Such changes were not observed in any of the HN2-treated animals, although there were small foci of karyorrhexis in the mucosa. Unfortunately, the sections from the HN2 groups did not always include the antral portion of the stomachs. It should be noted, however, that the gastric lesions described above with Myleran have not been observed by previous investigators in HN2-treated rats.

Discussion

The pathological changes observed in animals receiving HN2 were, in general, similar to those previously reported. However, the relationship between the extent of the changes produced and the dose given is worthy of comment. In some lymphoid tissues, such as the thymus, a maximum effect, that is, complete involution, was obtained at the LD_{50} level. Obviously, no greater change could have been produced by the administration of three times the LD_{50}. On the other hand, follicular damage in the spleen, to the point of almost complete disappearance, occurred only after the supralethal dose was given. At the LD_{50} there was only moderate reduction in the size of the splenic follicles.

In the case of the lymph nodes, damage varied, not only in relation to dosage, but also with respect to anatomical location. Although a marked decrease in the size of the nodes and a diminution in the number of lymphocytes occurred at the LD_{50} level, a substantial number of lymphocytes still remained. Only at supralethal levels (that is, three times the LD_{50}) did the destruction approach the so-called "depletion" or "atrophy" of lymphoid tissue that is generally considered to characterize the nitrogen mustards. Even then it was primarily the mesenteric lymph nodes that showed this extreme effect; the cervical nodes appeared to be somewhat more resistant.

Myleran, at the supralethal doses employed in this experiment, produced essentially the same pathological changes as those seen with HN2. However, two noteworthy distinctions may be mentioned. First, the maximum effects following Myleran were not seen until after the fourth day; in the case of HN2 the same degree of change was evident by the third day. Second, lymphoid damage from Myleran given at supralethal dosage was similar to that produced by the LD_{50} rather than the supralethal level of HN2.

In regard to the effects on bone marrow, nearly complete aplasia was already evident at 2 days following the 6 mg./kg. dose of HN2. At this time Myleran had caused only a 60 to 70 per cent depletion of nucleated elements. By 4 days the Myleran effect had become as severe as that produced by the supralethal dose of HN2. Following the LD_{50} of HN2, a moderate number of nucleated elements still persisted.

Severe changes occurred in one or more portions of the gastrointestinal tract in all groups of animals. Atrophy and erosions occurred in the small intestines within 3 days after the administration of either dose of HN2. Myleran given at 3 times the LD_{50} produced a less severe damage in the small intestines, which was maximal at 4 days. At 7 days this portion of the intestines was nearly normal in the Myleran animals. On the other hand, atrophy and erosions were profound in the large intestines of Myleran animals at 7 days. Similar damage to the colon was seen at 3 days in animals given the higher dose of HN2.

The present studies suggest that the small bowel is not as severely damaged by Myleran as by HN2. To determine this with certainty would require examination of animals at intervals more frequent than 2, 4, and 7 days. In the colon, the damage caused by both agents, given at 3 times the LD_{50}, was equally severe; however, the lesion developed more slowly in the Myleran animals. It is worth emphasizing that the lymphoid and bone marrow lesions produced by Myleran were similarly delayed.

We recognize that much remains to be determined concerning the intimate relationship between dosage and the minutiae of pathological change in each of the tissues susceptible to the actions of alkylating agents. Nevertheless, it is already clear that such dose-response relationships must be heeded before firm conclusions can be drawn regarding possible distinctions between the proximate modes of action of different polyfunctional alkylating compounds. The present observations are by no means unique. In 1948 it was already evident that compounds as closely related as sulfur mustard and HN2, as well as the aliphatic nitrogen mustards containing ethyl or β-chloroethyl groups in place of methyl, produced varying degrees of change in different susceptible tissues when each was given in the LD_{50} dose.[16, 17] Later polyethylenimines such as triethylene melamine were shown to cause a more severe aplasia in bone marrow at the LD_{50} than did a number of aliphatic nitrogen mustards.[4]

The combination of lymphoid, intestinal, and marrow damage provides a reasonable explanation for the fatality of animals treated with high doses of HN2. Clinical studies, on the other hand, indicate that the major limiting factor in the use of HN2 is bone marrow damage. No detectable gastrointestinal lesions have been observed following the systemic administration of this agent.[19] From early reports of clinical trials with Myleran, it appears that bone marrow damage is also the most important toxic effect.[20]

References

1. Philips, F. S. 1950. Pharmacol Revs. **2**: 281.
2. Gabriel, S. & C. F. von Hirsch. 1896. Ber. deut. chem. Ges. **29**: 2747.

3a. Ehrlich, P. 1910. The relations between chemical constitution, distribution, and pharmacological action. *In* Studies in Immunity. : 404–442. 2nd ed. Wiley & Sons. London, England.

3b. Biesele, J. J., F. S. Philips, J. B. Thiersch, J. H. Burchenal, S. M. Buckley & C. C. Stock. 1950. Nature. **166**: 1112.

4. Philips, F. S. & J. B. Thiersch. 1950. J. Pharmacol. Exptl. Therap. **100**: 398.
5. Goldin, A., H. A. Noe, B. H. Landing, D. M. Shapiro & B. Goldberg. 1948. J. Pharmacol. Exptl. Therap. **94**: 249.
6. Boyland, E., J. W. Clegg, P. C. Koller, E. Rhoden & O. H. Warwick. 1948. Brit. J. Cancer. **2**: 17.
7. Nickerson, M. 1949. Pharmacol. Revs. **1**: 27.
8. Philips, F. S., J. Scholler & S. S. Sternberg. Unpublished observations.
9. Hunt, C. C. & F. S. Philips. 1949. J. Pharmacol. Exptl. Therap. **95**: 131.
10. Ross, W. C. J. 1953. Advances in Cancer Research. **1**: 397.
11. Hunt, C. C. & F. S. Philips. Unpublished observations.
12. Burchenal, J. H. 1948. Radiology. **50**: 494.
13. Brand, E. D., T. D. Harris, L. S. Goodman & H. L. Borison. 1953. Federation Proc. **12**: 303.
14. Haddow, A. & G. M. Timmis. 1953. Lancet. **264**: 207.
15. Elson, L. A. 1955. Brit. J. Haematol. **1**: 104.
16. Graef, I., D. A. Karnofsky, V. B. Jager, B. Krichesky & H. W. Smith. 1948. Am. J. Pathol. **24**: 1.
17. Karnofsky, D. A., I. Graef & H. W. Smith. 1948. Am. J. Pathol. **24**: 275.
18. Kindred, J. E. 1947. Arch. Pathol. **43**: 253.
19. Spitz, S. 1948. Cancer. **1**: 383.
20. Galton, D. A. G. 1953. Lancet. **264**: 208.

HEMATOLOGICAL EFFECTS OF THE ALKYLATING AGENTS

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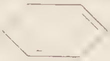
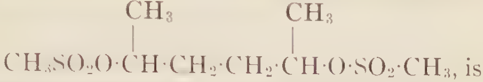
In the course of a general investigation of the nature of the growth-inhibiting action of carcinogenic and cytostatic compounds (Elson and Warren, 1947; Elson, 1948) it was noticed that the growth curves of rats treated with the alkylating agent Myleran, $CH_3{\cdot}SO_2{\cdot}O{\cdot}(CH_2)_4{\cdot}O{\cdot}SO_2{\cdot}CH_3$, closely resembled the curves obtained in response to whole-body X radiation, particularly with regard to the delayed effects. A delayed weight drop often occurred about twelve days after treatment with either Myleran or radiation. This delayed weight drop has been shown to be, in general, the result of anemia following the primary effect on the hemopoietic tissues occurring at the time of radiation or chemical treatment. The anemia is caused partly by inhibition of erythropoiesis, but chiefly by the development of a hemorrhagic state following thrombocytopenia.

The general pattern of weight response in the rat following whole-body irradiation has been described by Lamerton *et al.* (1953), and the relation of the blood changes to the various phases of radiation response, as indicated by the weight-response curves, has been discussed by Elson (1955a, 1956). Very briefly, all that need be mentioned here is that the initial weight-loss period, lasting for two to four days after irradiation, appears to be closely associated with a fall in the circulating lymphocytes and, in general, with the lymphoid effects of radiation. The delayed weight loss, while directly related to the thrombocytopenia, is associated primarily with effects on the bone marrow and with what we may conveniently refer to as the myeloid effects of radiation.

Investigation of the growth and blood effects of the nitrogen mustard derivative chlorambucil (CB 1348), $HOOC(CH_2)_3$$N(CH_2CH_2Cl)_2$, showed that this compound, in common with other nitrogen mustards and certain other types of alkylating agents, again was only partially radiomimetic in that it imitated mainly the lymphoid effects of radiation.

The complete hematological effects of radiation, however, can be imitated almost exactly by simultaneous administration of Myleran together with chlorambucil (FIGURE 1; Elson, 1955b).

Investigation of other members of the Myleran series of compounds, $CH_3SO_2{\cdot}O(CH_2)n{\cdot}OSO_2{\cdot}CH_3$, where *n* has been varied from 2 to 8, has shown that the blood-response curves following a single dose of the alkylating agent all show a distinct type of pattern characterized by a steady progressive fall in neutrophils to a minimum value, followed by an equally steady progressive recovery. Platelets are also depressed and, with doses just exceeding the lethal dose, death occurs only after an interval of at least 10 days; it is caused by massive hemorrhage or progressive anemia following thrombocytopenia.

Recently, a series of dimethyl Myleran compounds has been investigated; of these dimethyl Myleran itself,  $CH_3SO_2{\cdot}O{\cdot}\overset{CH_3}{\overset{|}{C}}H{\cdot}CH_2{\cdot}CH_2{\cdot}\overset{CH_3}{\overset{|}{C}}H{\cdot}O{\cdot}SO_2{\cdot}CH_3$, is about 3 times as active as Myleran in depressing neutrophils (FIGURE 2).

A comparison of the relative activity of the members of the Myleran and dimethyl Myleran series in depressing the number of circulating neutrophils in the rat is given in FIGURE 3 (see also discussion by G. M. Timmis, page 727). In both series, the peak of activity occurred in the compounds in which $n = 4$, but in every case the dimethyl Myleran compound was at least 2 to 3 times more active than the corresponding Myleran compound. Moreover, the members of the dimethyl Myleran series had a rather more rapid action, and the minimum values reached by the neutrophils occurred at 8 to 10 days instead of 12 to 14 days, as is usual in the Myleran series of compounds. Recovery to normal neutrophil counts was also correspondingly more rapid (FIGURE 2). Advantage is being taken of this rapidity of action and increased water solubility of dimethyl Myleran in clinical trials against leukemia.

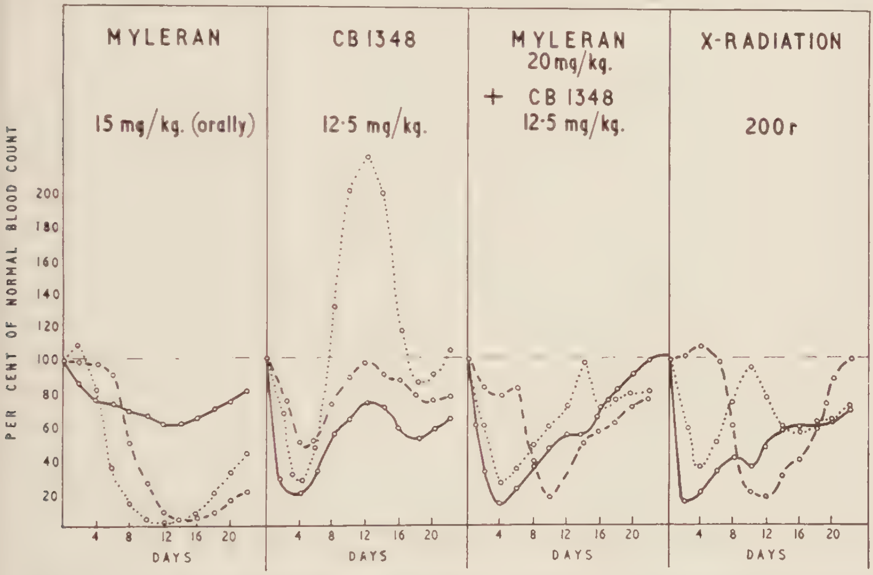


FIGURE 1. Blood response patterns in the rat to Myleran, chlorambucil (CB 1348), and to combined treatment with Myleran plus chlorambucil compared with the response to 200 r whole-body X irradiation. Symbols: ——— lymphocytes, ... neutrophils, and — — — — platelets.

Considering now the "aromatic nitrogen mustard" series of compounds, these again show a characteristic pattern of blood response to a single dose that is quite distinct from the pattern shown by the Myleran series. There was at first a rapid fall of both lymphocytes and neutrophils, with some fall in platelets. The most characteristic feature of the response, however, was

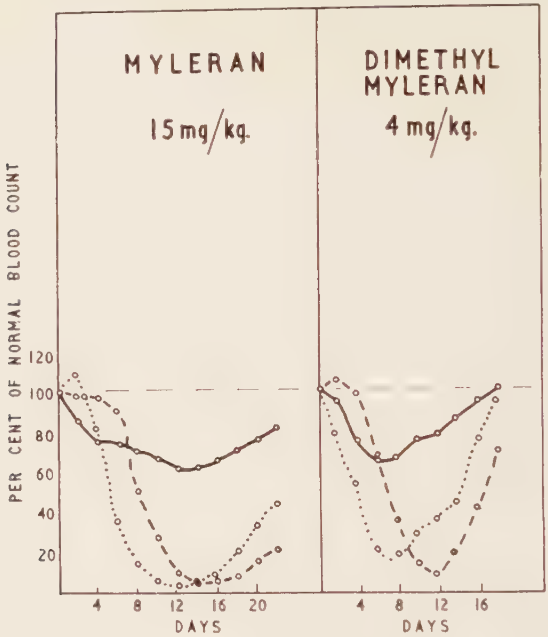
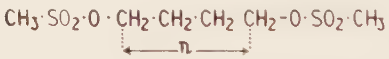
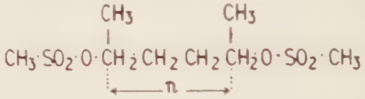


FIGURE 2. Blood response patterns in the rat to Myleran (15 mg./kg. orally) and t dimethyl Myleran (4 mg./kg. I.P.). Symbols the same as in FIGURE 1.



$$CH_3 \cdot SO_2 \cdot O \cdot \underbrace{CH_2 \cdot CH_2 \cdot CH_2\ CH_2}_{n} - O \cdot SO_2 \cdot CH_3$$

Myleran (n=4)



$$CH_3 \cdot SO_2 \cdot O \cdot \underbrace{\overset{CH_3}{\overset{|}{CH}}_2\ CH_2\ CH_2\ \overset{CH_3}{\overset{|}{CH}}_2}_{n} O \cdot SO_2 \cdot CH_3$$

Dimethyl Myleran (n=4)

n	Dose D. Mg/Kg.		Decrease % in Neutrophils from normal. F. (Minimum value reached)		F/D	
	Myleran series	Dimethyl Myleran series	Myleran series	Dimethyl Myleran series	Myleran series	Dimethyl Myleran series
2	200	50	0	0	0	0
3	40	7·5	50	92	1·3	12·3
4	8	3	70	75	8·8	25·0
5	12	5	85	80	7·1	16·0
6	40	10	60	70	1·5	7·0
7	60	30	40	98	0·7	3·3
8	80	40	50	98	0·6	2·5
9	100		40		0·4	
10	300		0		0	

FIGURE 3. Action of members of the Myleran and dimethyl Myleran series on circulating neutrophils in rats.

the very rapid recovery of circulating neutrophils, leading to a marked neutrophilia (up to 5 or more times the normal numbers) at about 8 to 10 days after a single dose (FIGURE 4). This neutrophilia appeared to be mainly the consequence of intense bone marrow regenerative activity following the initial short-term myeloid-depressing activity of the nitrogen mustard (Elson *et al.*, 1957). The bone marrow stimulation reached its peak 5 to 6 days after treatment with the drug, as has been confirmed by the finding that the proliferative activity of the bone marrow assessed by arresting mitoses in metaphase by the colchicine method was more than twice the normal value 5 days after a single dose of chlorambucil (G. Cardinali and L. A. Elson, unpublished).

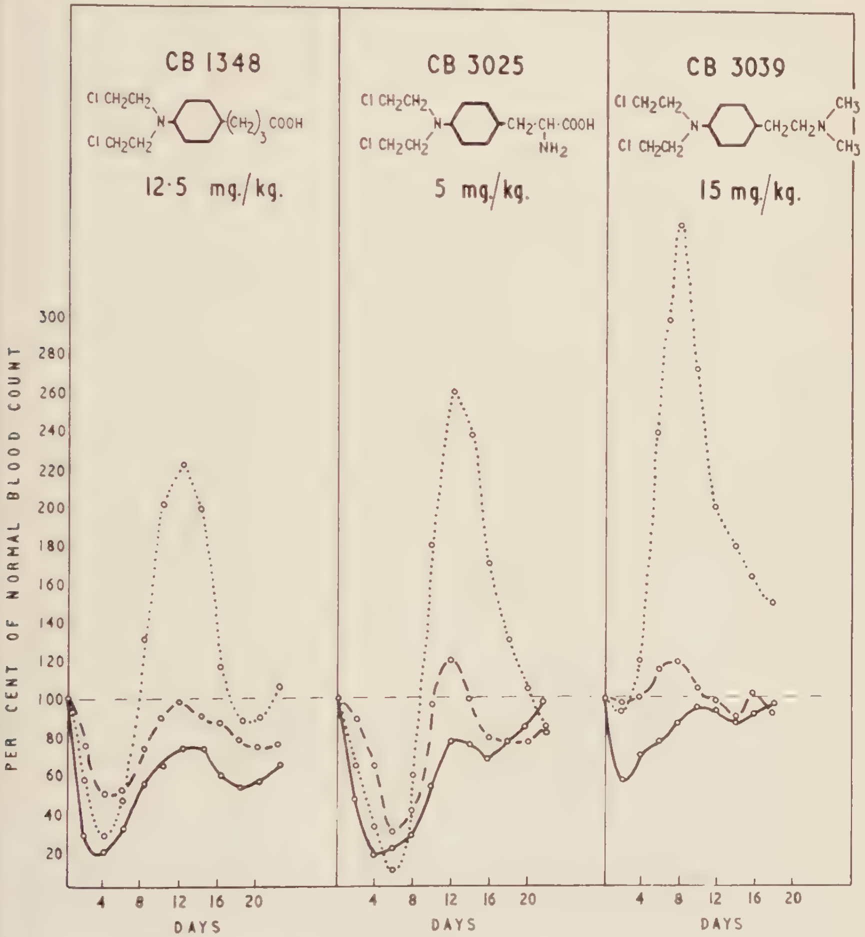


FIGURE 4. Blood response patterns in the rat to the "aromatic nitrogen mustard" derivatives CB 1348 (chlorambucil), CB 3025 (Melphalan), and CB 3039. Symbols the same as in FIGURE 1.

This "peak" in the blood-response curve to nitrogen mustards was not confined to the neutrophils, but was shown also, to a lesser extent, in the other blood cells, particularly the platelets (FIGURE 4), in accordance with the concept that it was a consequence of a general bone marrow stimulation.

A similar although less intense peak was seen also in the blood response curves to doses of whole-body X radiation (FIGURE 1), the peak again being most clearly demonstrated in the neutrophil response curves (Elson, 1957). Considering the blood response curves to Myleran and to chlorambucil, we see that the neutrophil peak in the chlorambucil response curve occurred a few days before the minimum value of the neutrophils was reached in the Myleran response curve. When doses of chlorambucil and Myleran were given simultaneously, the two drugs appeared to act independently of one another, and the modification of the chlorambucil peak by the slower neutrophil depressing action of Myleran produced a curve that resembled very closely that obtained in response to whole-body X irradiation. Analysis of the effects of this combined treatment with Myleran and chlorambucil administered simultaneously has revealed that some of the effects on the bone marrow are not as intense as in rats treated with the same dose of Myleran alone. Thus, under these conditions there was no additive toxicity effect of the drugs; indeed, the chlorambucil actually exerted a protective action against the Myleran effect. Thus, in 1 experiment in a group of 9 rats treated with a toxic dose of Myleran (20 mg./kg. by mouth), and at the same time given chlorambucil (12.5 mg./kg. I.P.), only 1 death occurred, whereas 7 deaths occurred in a group of 10 rats treated with Myleran (20 mg./kg.) alone. The protection was almost certainly due to the intense bone marrow activity that followed the initial depressive action of chlorambucil. Thus, this bone marrow stimulation, particularly the platelet formation that occurred just before the maximum depressive action of Myleran on platelets, counteracted the intense thrombocytopenia, the main cause of the toxicity of Myleran at this dose level. This combined treatment with Myleran (at a dose that, by itself, would be toxic) together with chlorambucil gave a blood-response curve that resembled very closely the curve in response to a dose of 200-r whole-body X radiation (FIGURE 1).

With regard to their action on the hemopoietic system, the nitrogen mustard series and the Myleran series of compounds each appeared to be capable of more selective action than X radiation and, with the Myleran series in particular, it was possible to reproduce, at the appropriate dose level, mainly the myeloid and some of the erythroid effects of radiation, with very little of its lymphoid effects. On the other hand, with the nitrogen mustards, although they showed some of the early myeloid effects of radiation, it was predominantly the lymphoid effects that were most closely imitated.

What exactly constitutes the difference in reactivity between the nitrogen mustard series of compounds and the Myleran series that could account for this difference in biological action, namely, the predominant action on lymphoid tissues shown by the mustards in contrast to the preferential selectivity of action on the myeloid tissues shown by the Myleran series, is still an open question. It cannot be accounted for by the different con-

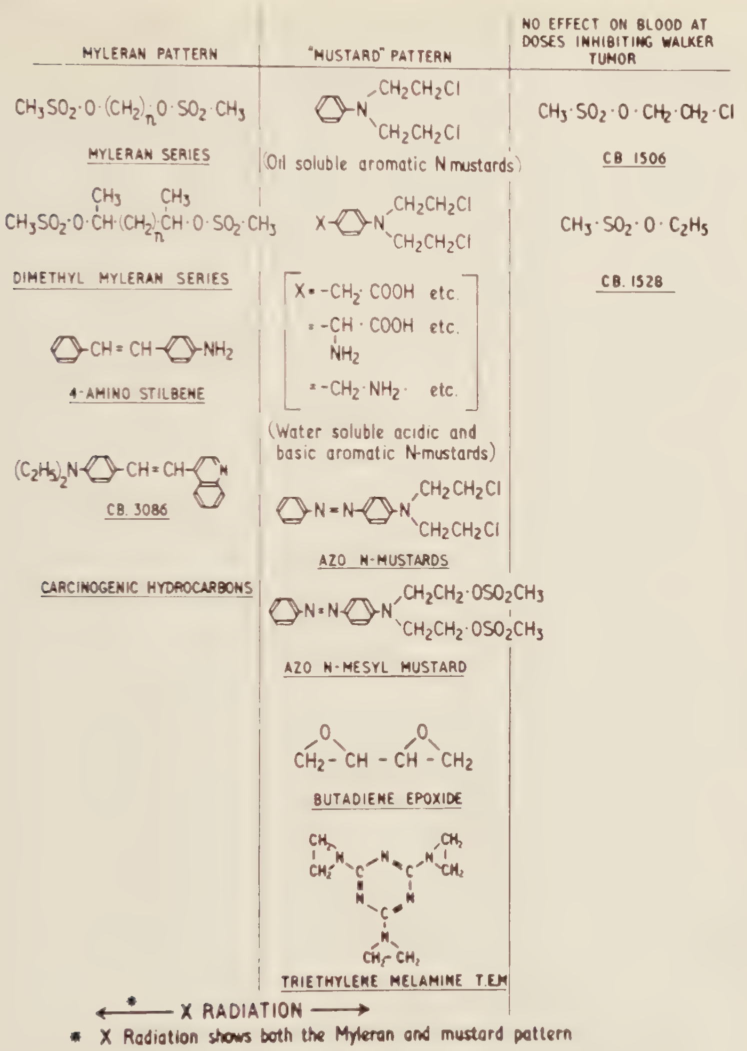
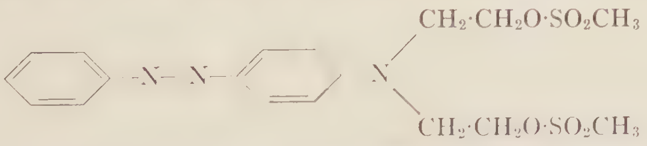

MYLERAN PATTERN
"MUSTARD" PATTERN
NO EFFECT ON BLOOD AT DOSES INHIBITING WALKER TUMOR
CH3SO2·O·(CH2)n·O·SO2·CH3
MYLERAN SERIES
(Oil soluble aromatic N mustards)
CH3·SO2·O·CH2·CH2·Cl
CB 1506
CH3SO2·O·CH(CH3)·(CH2)n·CH(CH3)·O·SO2·CH3
CH3·SO2·O·C2H5
DIMETHYL MYLERAN SERIES
CB. 1528
X = –CH2·COOH etc.
= –CH·COOH etc.
NH2
= –CH2·NH2· etc.
4-AMINO STILBENE
(Water soluble acidic and basic aromatic N-mustards)
CB. 3086
AZO N-MUSTARDS
CARCINOGENIC HYDROCARBONS
AZO N-MESYL MUSTARD
BUTADIENE EPOXIDE
TRIETHYLENE MELAMINE T.E.M
X RADIATION
* X Radiation shows both the Myleran and mustard pattern

Figure 5. Blood patterns of carcinogenic and cystostatic agents.

stitution of the chemically reactive groups, the Cl atom in the mustards and the mesyl group, $O{\cdot}SO_2CH_3$, in the Myleran compounds, for the compound



although containing the mesyl group of the Myleran series, shows a nitrogen mustard blood response pattern and not a Myleran pattern. The type of chemical reaction common to both groups of compounds is, of course, that of alkylation, and it seems reasonable to suppose that the differences in biological effects may be ascribed to differences in the mechanisms by which the alkylation occurs, for example, to action of the mustards chiefly by the S_N1-type mechanism and of the Mylerans chiefly by the more selective S_N2 type of mechanism.

It is now becoming clear, however, that the differences cannot be explained entirely by the classification of their reactions as S_N1 or S_N2 types as determined at any rate by the usual physical chemical methods (see papers by Price and Ross in part one of this monograph). By studying the hematological effects of various types of alkylating agents and carcinogenic and tumor-inhibiting substances, it has been possible, as shown in FIGURE 5, to assign a number of these to one or another of two classes, one group showing the blood response pattern characteristic of the nitrogen mustards and the other group showing the pattern characteristic of Myleran.

In the "Myleran-like" group, the most characteristic myeloid pattern was, of course, given by the Myleran and dimethyl-Myleran series, but 4-amino stilbene and 4-dimethyl-amino styryl quinoline (CB 3086), although showing some depression of lymphocytes, nevertheless showed predominantly the Myleran pattern of progressive neutrophil depression and thrombocytopenia leading to the characteristic hemorrhagic state and hemolymph changes. The carcinogenic hydrocarbons such as 1,2,5,6-dibenzanthracene, because of their insolubility and slow absorption, did not give as clear-cut a blood picture as Myleran; but here again the myeloid effects and characteristic hemolymph changes classified them with the Myleran rather than with the mustard series.

Among the alkylating agents, butadiene di-epoxide, which is believed to react exclusively by the S_N2-type mechanism, unexpectedly gave a mustard pattern and not a Myleran pattern. Triethylene melamine (TEM), also gave a mustard pattern.

Perhaps we should not ignore altogether the possibility that Myleran does not exert its biological action through an alkylating mechanism at all; Roberts and Warwick (1957) have shown that the monofunctional CB 1528, $C_2H_5{\cdot}O{\cdot}SO_2CH_3$, is capable of *in vivo* alkylation, at any rate with SH compounds (see discussion by J. J. Roberts, page 722), but this substance shows no radiomimetic effect on the blood (FIGURE 5). The most likely and certainly the most profitable approach at the moment, however, would seem to be on the lines of the suggestion of Timmis (1956) that if Myleran and, especially, dimethyl Myleran "acts biologically by alkylation, the action involves principally the relatively more powerful nucleophilic centres capable of adequately competing with water," coupled with the possibility that the Myleran type of compounds are to some extent selectively concentrated in myeloid tissues, particularly bone marrow.

References

ELSON, L. A. 1948. Acta Unio Intern. contra Cancrum. **6**: 396.

ELSON, L. A. 1955a. Symposium on Radiobiology. : 235. Butterworth. London, England.

Elson, L. A. 1955b. Brit. J. Haematol. **1**: 104.

Elson, L. A. 1956. V. Kongress der Europäischen Gesellschaft für Hämatologie. : 36. Springer-Verlag. Berlin, Germany.

Elson, L. A. 1957. Advances in Radiobiology. 5th Intern. Conf. Stockholm, Sweden. : 372.

Elson, L. A., D. A. G. Galton, L. F. Lamerton & M. Till. 1957. Progress in Radiobiology. 4th Intern. Conf., Cambridge, England. : 285.

Elson, L. A. & F. L. Warren. 1947. Brit. J. Cancer. **1**: 86.

Lamerton, L. F., L. A. Elson & W. R. Christensen. 1953. Brit. J. Radiol. **26**: 510.

Roberts, J. J. & G. P. Warwick. 1957. Nature. **179**: 1181.

Timmis, G. M. 1956. Brit. Empire Cancer Campaign Ann. Rept. 1955. : 37.

DISTRIBUTION AND FATE OF ALKYLATING AGENTS*

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Studies on the metabolism of alkylating agents have been prompted not only by the usual need to know more of their absorption, distribution, and excretion, but by the hope that such information may give some insight into their mechanism of action. A review of the early work on their pharmacology has been published (Philips, 1950).

Because of their extremely high toxicity and the lack of methods sufficiently sensitive for their qualitative as well as quantitative determination, usually it has been necessary to synthesize these drugs with radioisotope labels.

Selection of the proper label and its position in the drug molecule are important if one is to obtain results that will be useful in interpreting the mechanism of action.

Sulfur Mustard (Bis-β-Chlorethyl Sulfide)

One of the earliest of the alkylating agents to receive attention was sulfur mustard. The observation that certain thiophosphoramides are carcinostatic and clinically effective has renewed interest in this compound (Wheeler *et al.*, 1955; Alpert and McCabe, 1957; Davison *et al.*, 1957).

A number of years ago Boursnell *et al.* (1946) injected the sulfur-labeled compound into rabbits and studied its distribution in the tissues. Much of the radioactivity was excreted in the urine, but a significant amount was present in the bile. Most of that in the tissues was believed to be combined with protein.

Davison *et al.* (1957) recently have synthesized sulfur mustard from radioactive hydrogen sulfide by interaction at low pressure with ethylene oxide to yield thiodiglycol, which forms sulfur mustard upon treatment with hydrochloric acid (FIGURE 1).

The urinary metabolites of this substance have been investigated in the mouse, the rat, and in two human subjects. Essentially all of the drug was excreted by the small animals in 3 days: more than half emerging in the first 6 hours, and nearly 90 per cent in the first day. About half was excreted by the human in the course of 2 days, but the dose employed was about one twentieth of that given the animals. Chromatography on Dowex 1 anion-exchange resin revealed the presence of at least 6 compounds, the neutral peak accounting for about 20 per cent of the total (FIGURE 2). All the urinary metabolites were either neutral or anionic in nature. Essentially the

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same pattern was seen in chromatograms using urine samples collected at different intervals after administration of the drug and in all three mammalian species tested. Using two different derivatives, isotope dilution techniques revealed that approximately 10 per cent of the neutral fraction was thiodiglycol. The nature of the other neutral substances is unknown,

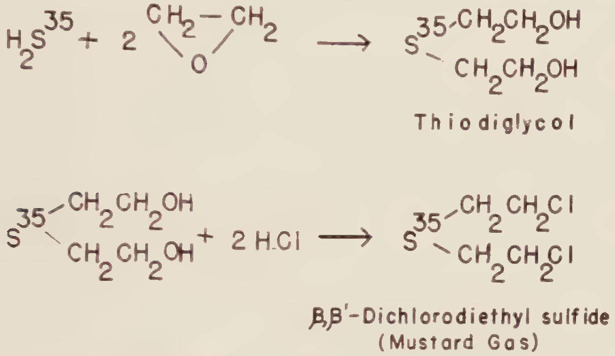

FIGURE 1. Scheme for the synthesis of sulfur mustard-S^{35}.

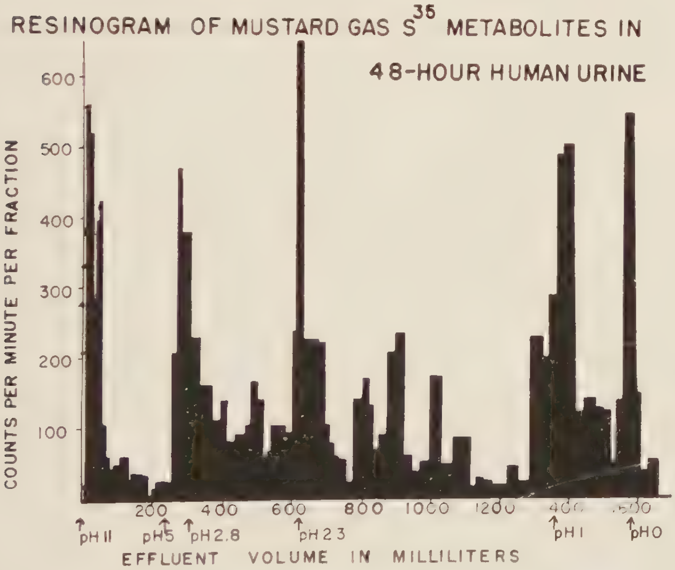

FIGURE 2. Radioactivity of urinary metabolites after the administration to a patient of sulfur mustard-S^{35}.

but experiments with various hydrolytic methods are in progress to test for the presence of a conjugate. Because of the obvious acidic nature of substances eluted by acid (*p*H 1 or below), studies have been made of the possible oxidation of mustard to sulfate or some sulfonic acid. The peak eluted with *p*H 0 acid has been found to contain, not only traces of sulfate, but also two sulfonic acids, isethionic and sulfoacetic acid. The close relationship of these latter two compounds is apparent from their chemical structures (FIGURE 3). Thiodiglycolic acid would appear to be a logical intermediate between thiodiglycol and sulfoacetic acid, and about 1.5 per cent of the radioactivity could be accounted for as a derivative of this acid that was prepared from whole urine. This substance was eluted with *p*H 1 acid. The nature of the less acidic substances is unknown.

After intravenous administration to patients, 80 per cent of the drug disappeared from the blood in less than 1 minute, and the portion remaining was quite stable, disappearing very slowly over a period of days. Less than 2 per cent of the drug was found in the red cells.

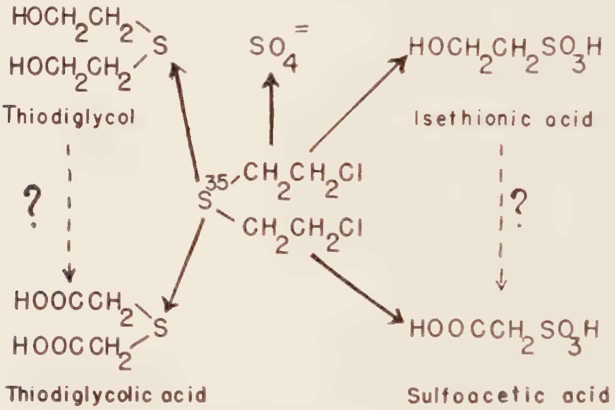

FIGURE 3. Scheme for the metabolism of sulfur mustard.

Because of previous reports that sulfur mustard was excreted in the bile and the possibility that it might be present there as taurine, preliminary experiments were made upon biliary excretion in the rat. While appreciable amounts of radioactivity were found in the bile, paper chromatography provided no evidence of its conversion to either taurine or taurocholate.

Nitrogen Mustard (Mechlorethamine Hydrochloride, HN2)

Skipper and his co-workers (1951) studied the metabolism of C^{14}-nitrogen mustard labeled in the methyl position. It was injected into normal and leukemic mice in doses of 4.0 mg. per kg. Between 10 and 18 per cent of the methyl carbon was oxidized to carbon dioxide and was exhaled. A small amount of unidentified basic substance containing the active methyl carbon also was exhaled. As was to be expected, the radioactivity was distributed throughout all organs and tissues. The specific activity of the blood serum was higher than that of the cells. The intestine was consistently the tissue of highest activity in normal mice. The results showed that the turnover of C^{14} from methyl-labeled nitrogen mustard in various tissues was slow and the fixation in tissues was high, being about 30 to 50 per cent in 6 hours. Perhaps the most interesting observations were the unexpected low activities in the bone marrow and the preferential deposition in the lymph nodes, adrenals, thymus, and brain of leukemic animals as compared with nonleukemic controls.

In more recent studies in our laboratories (Nadkarni *et al.*, 1956) a rapid decrease in radioactivity in blood occurred when animals were injected with methyl-C^{14}-labeled HN2. Over 90 per cent of the isotope was removed from blood within 30 seconds. Of the radioactivity remaining in the blood, almost all was present in the plasma.

The question arose as to whether the radioactivity assayed in biological specimens was due to the intact drug or to products formed as a result of transmethylation from the labeled HN2. For this purpose a study was made of the incorporation of radiocarbon into choline, creatine, and the *S*-methyl group of methionine isolated from the livers of rats injected previously with HN2 (Trams and Klopp, 1955). Rats were injected intravenously with a total dose of 1.0 mg. per kg. of body weight of the methyl-labeled material. They were sacrificed 24 hours after the last injection.

The radiocarbon isolated from liver phospholipid choline amounted to less than 0.5 per cent of the total injected radioactivity. Calculations of the total body choline content in rats indicated that the total radiocarbon contained in this compound did not exceed 4 per cent of the total injected dose. An increase in the specific activity of choline in animals receiving isotopic HN2 over a period of 2 or 4 days in smaller doses suggested that a higher incorporation of radiocarbon was obtained by repeated injections of small fractions of HN2-C^{14} as compared with the administration of one larger dose only. There was little or no radiocarbon in the creatine or the *S*-methyl group of methionine. Failure to obtain appreciable amounts of C^{14} in creatine or in the *S*-methyl group of methionine, despite incorporation of radiocarbon into choline, may have been due to inhibited transmethylation from choline into other methyl acceptors, such as methionine. There was evidence to indicate that HN2 inhibited the choline oxidase systems (Barron *et al.*, 1948) and therefore may have blocked further transfer. It was concluded that, although a substantial amount of the drug was demethylated, as indicated by the large fraction exhaled as carbon dioxide, transmethylation did not represent a major metabolic pathway for the drug.

Further experiments were conducted to determine whether the *N*-methyl group of HN2 was liberated and subsequently oxidized to formaldehyde (Trams and Nadkarni, 1956). Liver homogenates from rabbits or rats were prepared, with slight modifications, according to a method described by Axelrod (1956). Flasks were charged with the enzyme system, magnesium chloride, triphosphopyridine nucleotide, semicarbazide, and substrate. Formaldehyde was determined colorimetrically by the chromotropic acid reaction. Added formaldehyde, carried throughout the procedure as control, was recovered in yields of from 80 to 85 per cent. Whole liver homogenates were less effective in dealkylation than the supernate fraction, especially that of rat-liver homogenates. The relative amounts of formaldehyde obtained from the *N*-methyl group of HN2 decreased at higher substrate concentrations. The results of a representative experiment are illustrated in FIGURE 4. Incubation of benadryl with varying amounts of HN2 added showed that the demethylation-oxidation proceeded uninhibited in the presence of HN2, indicating that the relative decreased yield of formaldehyde from HN2 at higher substrate concentrations was not due to self-inhibition. It was observed that the bis-(β-hydroxyethyl) analogue of HN2 yielded formaldehyde in amounts comparable to those from HN2.

The ability of enzyme preparations from rat hepatoma "Novikoff" to demethylate HN2 was low in comparison to that of normal liver from rats of the same strain.

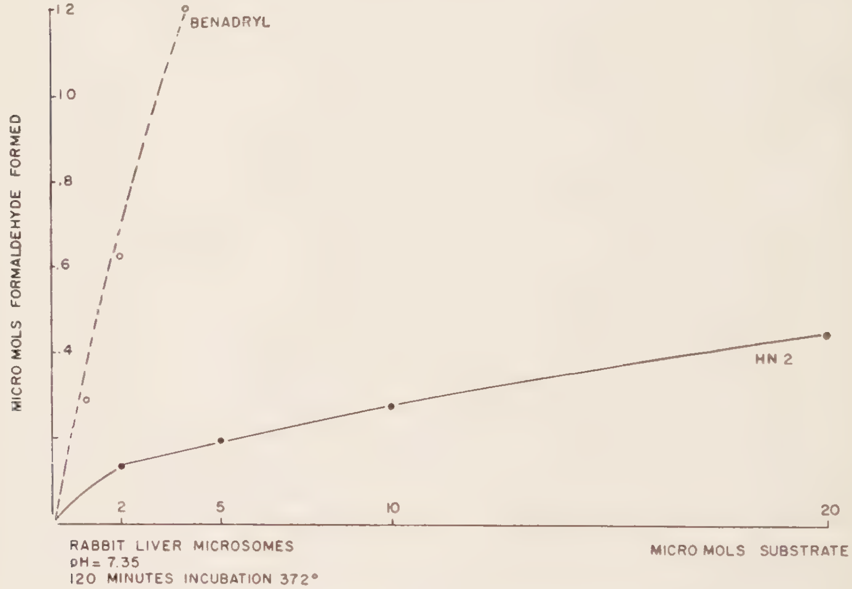

FIGURE 4. N-demethylation of diphenhydramine (Benadryl).

The results indicate that the drug was readily demethylated by an enzyme in the particulate and soluble supernate fraction of liver homogenates prepared by high-speed centrifugation. Axelrod (1956), who studied the demethylating capacity of this preparation on various other substrates, has shown that the enzyme itself appeared to be localized within the microsomes, while the soluble supernate probably contained necessary cofactors.

In most experiments described here the substrate concentrations exceeded those usually obtained with therapeutic doses *in vivo*. It was estimated that almost all of an LD_{50} dose of HN2 injected intravenously into an adult rat would be demethylated within 1 hour. This would account readily for the appreciable amount of carbon dioxide-C^{14} eliminated following the injection of methyl-C^{14}-labeled HN2.

In a search for other pathways for the metabolism of the β-chlorethyl portion of the molecule, the possibility of glycolaldehyde formation was investigated. No glycolaldehyde from HN2 could be demonstrated. However, the recovery of added glycolaldehyde from the incubation mixture was not satisfactory and, therefore, the presence of this intermediate remains unconfirmed. Subsequent studies with respect to the cleavage of the *N*-ethyl linkage showed that acetaldehyde could be detected in the incubation mixture when HN2 was added as the substrate. The yield of acetaldehyde from HN2 was better at relatively low concentrations of HN2. Recovery of acetaldehyde from other compounds containing the *N*-ethyl group was obtained in good yield. Compounds containing the *N*-β-hydroxyethyl group failed to yield acetaldehyde upon incubation. When *N*,*N*-diethylnicotinamide (Coramine) was incubated, together with various concentrations of HN2, additive amounts of acetaldehyde were formed when the concentration of added HN2 was low. At higher concentrations of HN2, however, the yield of acetaldehyde from Coramine was markedly decreased. This is illustrated in FIGURE 5.

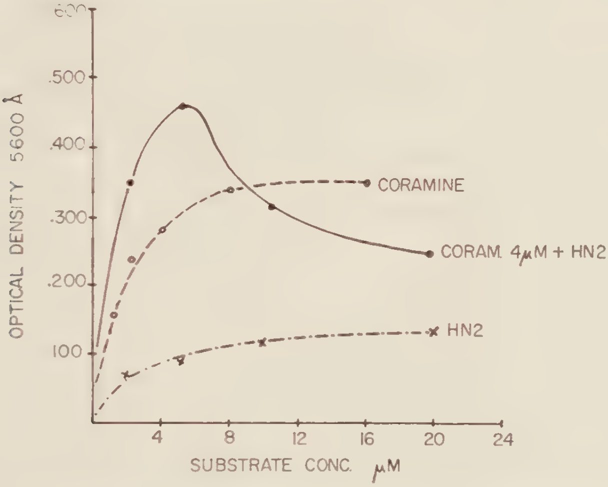

FIGURE 5. *N*-de-ethylation of nikethamide (Coramine), mechlorethamine (HN2), and a combination of the two.

Somewhat incongruous is the finding that acetaldehyde was formed with HN2 as the substrate, since complete reduction of the β-carbons of HN2 seemed unlikely without postulating a rearrangement through an allyl configuration. Two other mechanisms could be visualized in the formation of acetaldehyde from HN2: (1) removal of the *N*-ethyl groups by reductive cleavage and (2) hydrolysis at the *N*-C linkage with the formation of *N*-alkyl-substituted hydroxylamine. The latter hypothesis was of some interest, since such *N*-alkyl-substituted hydroxylamines or their degradation products similar to hydroxylamine might have been toxic to the choline esterase and choline oxidase systems. The formation of such compounds might explain also the acute neurotoxic effects of nitrogen mustards observed after the administration of supralethal doses.

These data indicate that tracer studies with N-methyl-C^{14}-labeled HN2 may lead to results that do not necessarily represent the uptake or distribution of the whole HN2 molecule. This appears to be especially true of any analyses made a long time after drug administration.

Chromatographic analysis of urine after the administration of HN2-C^{14} revealed the presence of at least eight labeled products. Some of these were primary or secondary amines and were not identified further.

Triethylenephosphoramide-P^{32}

Two groups of investigators have studied the fate of triethylenephosphoramide-P^{32} (TEPA) in animals. Nadkarni *et al.*, (1953, 1957a) obtained the material labeled with P^{32} from the research laboratories of the Calco Chemical Division, American Cyanamid Company, Pearl River, N. Y. The chemical and radio purity of the compound were confirmed by paper chromatography. The drug was administered to groups of tumor-bearing and control mice, and the tissue distribution and excretion of radioactivity were determined.

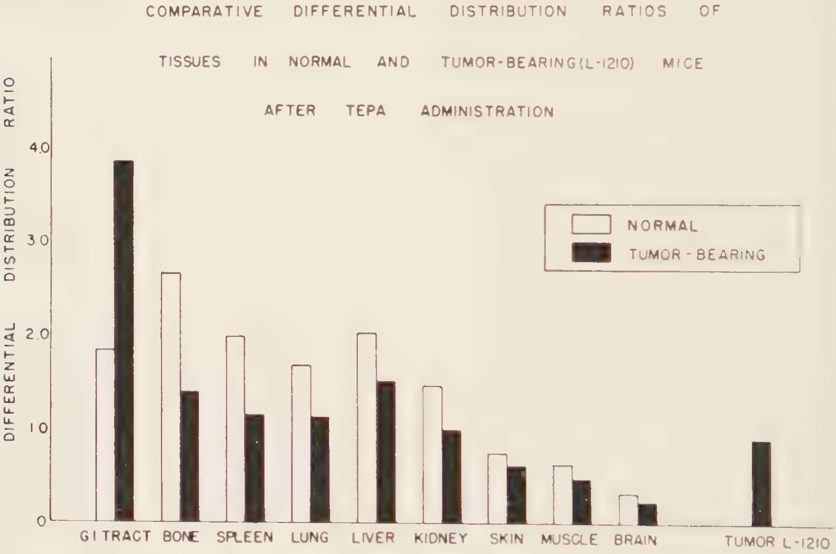

FIGURE 6. Distribution of radioactivity in tissues 24 hours after intraperitoneal administration of TEPA-P^{32}.

The differential distribution ratios (DDR) for the various organs and tumors were calculated as follows:

$$\text{DDR} = \frac{\text{Residual radioactivity per gram of organ tissue}}{\text{Residual radioactivity per gram of body weight}}$$

A ratio greater than one denotes selective concentration. FIGURES 6, 7, and 8 show the differential distribution ratios of various organs of control mice compared with those of tumor-bearing animals. There was no marked

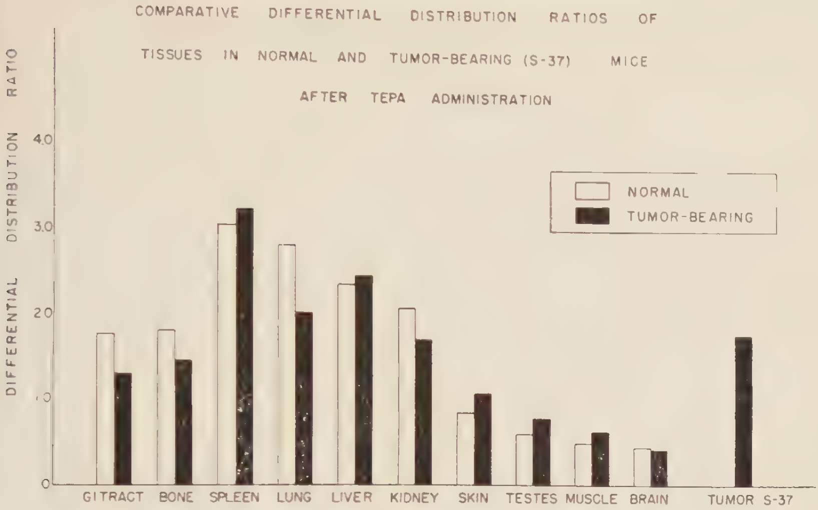

FIGURE 7. Distribution of radioactivity in tissues 24 hours after intraperitoneal administration of TEPA-P^{32}.

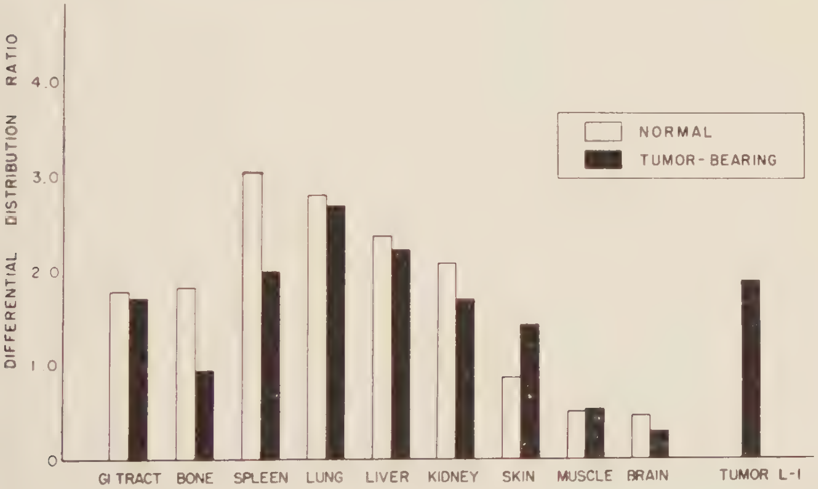

FIGURE 8. Distribution of radioactivity in tissues 24 hours after intraperitoneal administration of TEPA-P^{32} (lymphosarcoma 1).

selective localization of radioactivity in the various organs of tumor-bearing mice as compared with that in control animals. While several tissues of mice bearing sarcoma 37 or lymphosarcoma 1 showed selective concentrations, the relative values were not very different from those in control animals. In mice bearing leukemia 1210, the gastrointestinal tract showed a concentration nearly twice that of the controls. The relative uptake of radioactivity per gram of tumor tissue and the differential distribution ratios of the tumor under study are shown in FIGURE 9. The specific activities of the three tumors were approximately in the same order as their known susceptibility to the drug.

Following intraperitoneal administration of TEPA-P^{32} to control and tumor-bearing mice, between 60 to 80 per cent of the radioactive phosphorus was excreted within 24 hours. The major path of excretion was through the kidneys, with little radioactivity in the feces.

Of the two urinary metabolites separated by paper chromatography, one with the lower R_f value represented 80 per cent of the urinary radioactivity and corresponded to inorganic phosphate derived from completely hydrolyzed TEPA-P^{32}. The R_f value of the remaining metabolite, upon acid or alkali treatment, changed to that of inorganic phosphate. It seemed probable that the second metabolite was an intermediate between the triethylene phosphoramide and its final hydrolytic product, inorganic phosphate. No unchanged TEPA was detected in the urine.

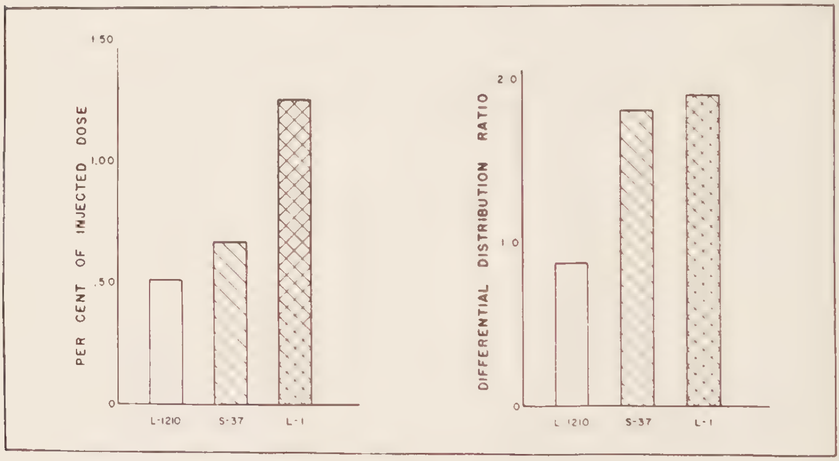

FIGURE 9. Distribution of radioactivity in three types of tumors 24 hours after administration of TEPA-P^{32}.

Craig and Jackson (1955) studied the distribution of labeled TEPA after its injection into rats bearing the Walker carcinosarcoma or Jensen sarcoma. No specific localization of drug occurred in any tissue examined. Over 80 per cent of the injected dose was excreted in the urine in 24 hours. Most of this was attributed to unchanged drug. This finding appears incongruous in view of the postulated high degree of chemical and biological reactivity of this group of alkylating drugs. However, it should be recalled that Nadkarni *et al.* employed mice, and Craig and Jackson used rats.

Oxapentamethylene Diethylenethiophosphoramide

A preliminary study of the metabolism of *N*-(3-oxapentamethylene)-C^{14}-*N′*,*N″*-diethylenethiophosphoramide (OPSPA) has been reported by Maller and Heidelberger (1956). Examination of the urinary metabolites of OPSPA indicated that its fate in tumor-bearing rats and human breast carcinoma patients was similar. Carrier crystallization analyses of these urines and aqueous-soluble fractions of blood plasma showed that only a small percentage of the radioactivity was due to unchanged OPSPA. There was evidence of appreciable metabolic cleavage of the sidechain to morpholine, which increased with time after administration.

The desulfurated derivative of OPSPA, *N*-oxapentamethylene-*N′*,*N″*-diethylenephosphoramide (MEPA), 24 hours after injection of OPSPA was present to the extent of 66 per cent in rat urine and 25 per cent in human urines. Human plasma drawn 1 and 12 hours following intravenous injection respectively contained 72 per cent and 55 per cent of the circulating radioactivity as MEPA. The water-soluble fractions from rat liver and Flexner-Jobling carcinoma taken 2 hours after injection of OPSPA respectively contained 28 per cent and 58 per cent of the radioactivity as MEPA. This compound was strongly tumor-inhibitory, but not as potent as OPSPA in the rat. Thus, these investigators demonstrated the presence of an active tumor-inhibitory compound in the tissues.

Triethylenimino-s-Triazine-C^{14} (*Triethylene Melamine*)

Radioactive triethylene melamine (TEM), with the three carbon atoms of the triazine ring labeled with C^{14}, was synthesized in an over-all yield of 22 per cent of theory from urea-C^{14} by methods described previously in the literature with modifications to suit preparation on a semimicro scale (FIGURE 10).

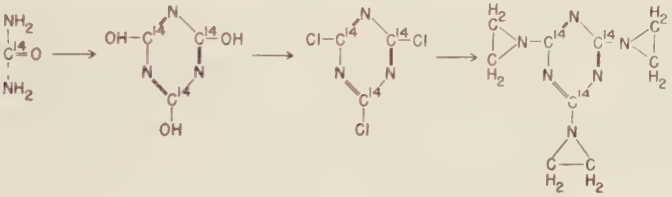
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FIGURE 10. Scheme for synthesis of triazine-labeled TEM-C^{14} from urea-C^{14}.

When injected into mice there was little uptake of radioactivity by most organs of either the control or the tumor-bearing animals. There was no evidence of a striking difference in distribution between any of the tumor-bearing groups and the corresponding control groups. The gastrointestinal tract had a relatively higher concentration of radioactivity than most tissues (Nadkarni *et al.*, 1954). A comparison of the differential distribution ratios of the tumor tissues (FIGURE 11) indicated that no selective concentration of radioactivity occurred in the types of tumors studied. These results agree with those mentioned by Skipper (1953), who has included ring-labeled TEM among compounds that did not show preferential localization in cancer cells.

Between 72 and 88 per cent of the injected radiocarbon was excreted within 24 hours. Differences among the various tumor-bearing and control groups were so small as to be of doubtful significance. In all cases, most of the radioactivity was found in urine, and only 1 to 2 per cent in exhaled carbon dioxide. The rate of respiratory excretion decreased rapidly after the first 2 hours, and no activity could be found during the 24th hour. Chromatography of the urinary metabolite, with original TEM-C^{14} co-chromatographed as a control, revealed a sharp difference between the R_f value of TEM-C^{14} and that of the metabolite. There was no radioactive peak corresponding to the R_f value of unchanged TEM-C^{14}. Alkali treatment had no effect on the R_f value of the urinary metabolite, but it changed the TEM to a product having the same R_f value as that of the metabolite. By isotope dilution technique it was demonstrated that this metabolite was cyanuric acid. No injected TEM was excreted unchanged.

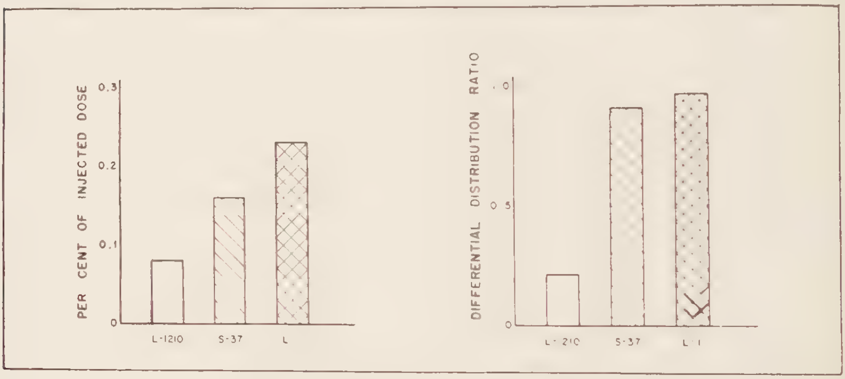

FIGURE 11. Distribution of radioactivity in three types of tumors after administration of triazine-labeled TEM.

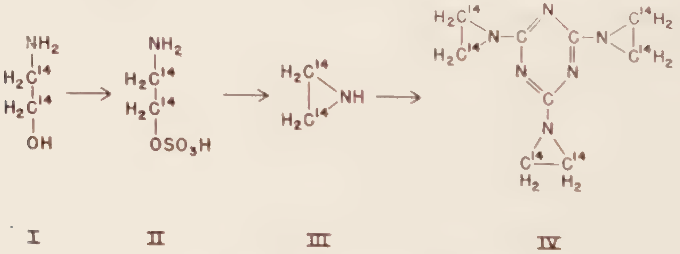

FIGURE 12. Scheme for synthesis of ethylenimino-labeled TEM-C^{14} from ethanolamine-C^{14}.

Triethylene-C^{14}-Imino-s-Triazine (Triethylene Melamine)

TEM with carbon-14 in the ethylenimino ring was synthesized in this laboratory by methods described in the literature as modified for use with radioactive materials (FIGURE 12). The yield was 11 per cent of theory, with an estimated specific activity of 1.1 μc. per mg.

When the material was injected intravenously into patients or animals, most of it disappeared rapidly from the blood (FIGURE 13; TABLE 1). After an initial selective uptake, the specific activities of the kidneys, livers, and lungs of rats became fairly constant 15 minutes after injection (FIGURE 14).

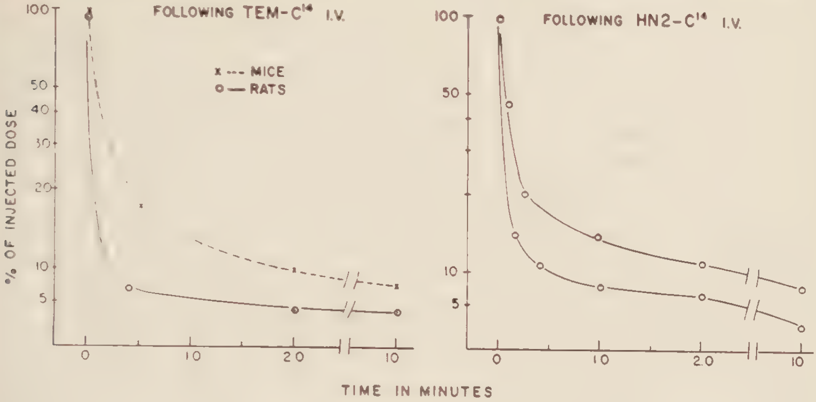

FIGURE 13. Rate of disappearance of radioactivity from the blood of animals after the administration of ethylenimino-labeled TEM or methyl-labeled HN2.

After intraperitoneal injection into mice, between 68 and 73 per cent of the injected radioactivity was excreted in the urine within 24 hours, and only a small percentage was excreted in the following 24 hours. Comparatively little radioactivity was found in the feces and in exhaled carbon dioxide. Chromatographic separation on an ion exchange column revealed at least 16 urinary metabolites (FIGURE 15). Five major radioactive peaks in the eluent accounted for 74 per cent of the total excreted radioactivity, the largest being 34 per cent. Less than 1 per cent of the urinary radioactivity was identified as urea (Goldenthal *et al.*, 1956). One of the major peaks was identified as creatinine. Later experiments in patients failed to reveal significant amounts of radioactive creatinine in the urine after the administration of ethylenimino-labeled TEM (Trams and Nadkarni, 1956).

TABLE 1
RADIOACTIVITY IN PATIENTS FOLLOWING ADMINISTRATION OF ETHYLENIMINO-LABELED TEM-C14 I.V.

1. Patient G. F.: 30-yr.-old male, acute lymphatic leukemia. Injected dose: 2.0 mg. = 1,440,000 cpm

Blood level in per cent of injected dose

3 Min.	7.9%
3 Hours	3.9%
6 Hours	0.8%
12 Hours	0.8%
24 Hours	0.8%

2. Patient J. T.: 59-yr.-old male, epidermoid carcinoma of the larynx. Injected dose: 2.2 mg. = 1,600,000 cpm

Blood level in per cent of injected dose

3 Min.	7.8%

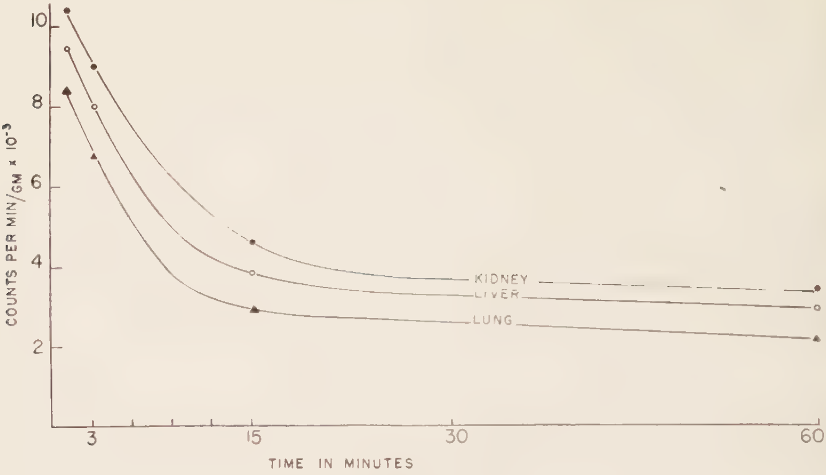

Figure 14. Specific radioactivities of kidney, liver, and lung after the I.V. administration of ethylenimino-labeled TEM-C^{14} to rats.

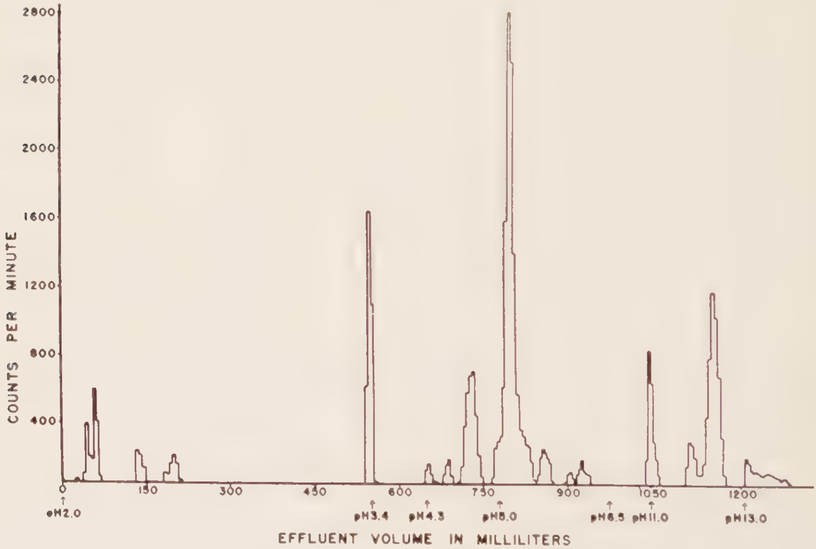

Figure 15. Ion exchange chromatogram of the radioactivity of urinary metabolites after the administration of ethylenimino-labeled TEM-C^{14} to mice.

Since it had been demonstrated that, upon alkaline hydrolysis, TEM yielded cyanuric acid and ethanolamine, it seemed possible that this same reaction occurred *in vivo*, and that the numerous radioactive peaks in the urine of subjects administered ethylenimino-labeled TEM-C^{14} were due to the metabolism of ethanolamine. To test this hypothesis, carbon-14 ethanolamine was administered to mice in a comparable molecular ratio.

The metabolites were chromatographed with the same resin and eluted with the same buffers as those used with the metabolites of TEM-C^{14}. It may be seen from FIGURE 16 that the chromatogram was substantially different from that obtained with the metabolites of TEM.

On the assumption that any two-carbon fragment derived from the ethylenimino moieties of TEM would enter the metabolic pool *in vitro*, studies were performed to trap and determine such fragments in the aldehyde form. As expected, there was no formaldehyde formed in liver homogenates with TEM as the substrate, although the experimental conditions were varied over a wide range of *p*H and incubation period. If the removal of the two-carbon fragments from the nitrogen took place analogous to the *N*-demethylation of HN2, glycolaldehyde would have been the expected product. No glycolaldehyde could be detected, but the recovery of added glycolaldehyde was not satisfactory. Formation of acetaldehyde was not demonstrated in experiments similar to those employed with HN2.

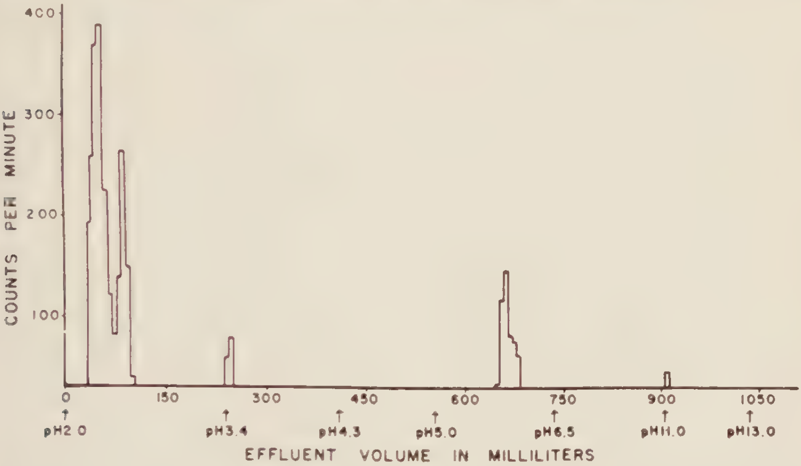

FIGURE 16. Ion exchange chromatogram of the radioactivity of urinary metabolites after the administration of ethanolamine-C^{14} to mice.

The possibility that glycine was formed as an intermediate was increased by the isolation of radioactive creatinine from the urine of mice injected with TEM-C^{14}. A number of rats received either glycine-C^{14} or TEM-C^{14} followed by benzoate. Also, one patient was injected intravenously with TEM-C^{14} following the oral administration of sodium benzoate. The proportion of radiocarbon recovered in urinary hippuric acid following the administration of TEM-C^{14} was so low that the formation of glycine-C^{14} as a major metabolic intermediate in the degradation of TEM *in vivo* appeared unlikely.

Other studies in this laboratory had shown that compounds that utilize acetate as precursor, such as fatty acids, isolated from TEM-C^{14}-administered animals were very low in radioactive content. In other experiments the radioactivity of nucleotides, for which glycine serves as a known precursor, was too low to substantiate further the hypothesis of the conversion of the ethylenimine-C^{14} moiety of TEM to glycine. These results offer almost no clues as to the major metabolic pathways of the ethylenimino moiety of triethylene melamine.

Busulfan-S^{35} (Myleran-S^{35},1-4,Dimethanesulfonoxybutane)

Busulfan was synthesized labeled with sulfonoxy-S^{35}. The distribution was studied following intravenous administration. In analogy to the other alkylating agents studied, there was a rapid disappearance of isotope from the blood following injection (Trams *et al.*, 1957). Most of the radiosulfur from the drug was concentrated in the liver, kidneys, and small intestines of the rat immediately following the injection. There was no selective concentration of isotope in any one organ at 24 hours after administration. From 60 to 85 per cent of the injected radioactivity, depending upon the route of administration, was excreted into the urine, chiefly in the form of methanesulfonic acid.

Busulfan-C^{14} (Myleran-C^{14},1-4,Dimethanesulfonoxybutane)

Busulfan was synthesized with C^{14} in the 1:4 carbons of the butane moiety (Trams *et al.*, 1957). The specific activities of the kidneys, liver, small intestine, and lungs were higher than those of any other organs at any time following intravenous injection into rats. In the liver only small amounts of radioactivity were associated with proteins and fats. The radioactivity of the sodium nucleates of liver was very low. Within 24 hours approximately 12 per cent of the radioactivity was excreted as carbon dioxide. From 25 to 30 per cent was excreted into the urine and 8 per cent in the feces.

Within 6 hours after the injection, up to 50 per cent of the urinary radioactivity was present as unchanged busulfan. The presence of at least 14 radioactive compounds in the urine was demonstrated by ion exchange chromatography. Two of these, representing from 70 to 80 per cent of the total urinary C^{14}, were neutral, volatile compounds.

The drug showed a rapid disappearance from the blood of cancer patients, over 90 per cent of the injected isotope being removed within 2 to 3 minutes. Most of the residual activity was associated with the plasma (Nadkarni *et al.*, 1957b).

Summary

The radioactivity of labeled alkylating agents disappeared rapidly from the blood with maintenance of a low and constant level of radioactivity for about 48 hours.

The more reactive alkylating drugs presumably interacted with target molecules immediately after administration. These reaction products were then metabolized or excreted over a period of several days.

The radioactive metabolites appearing when the triazine, methanesulfonate, or phosphate portions of the drug were labeled indicated that the carrier moiety was separated readily from the alkylating groups. Numerous metabolites appeared when the ethylenimino group of TEM or the butane moiety of Myleran was labeled. This was true also in the case of S^{35}-sulfur mustard, indicating that, in the case of this drug, there was no appreciable separation of the alkyl groups from the sulfur bridge.

Since transformation of the ethylenimino or butane moiety into common 2- and 1-carbon fragments with incorporation of C^{14} into creatinine, urea, glycine, etc., occurred to a small extent only, it is postulated that most of the urinary constituents represented alkylated derivatives of normal metabolites.

The quantitative difference in the excreted metabolic products in experimental animals and human patients may have been due to the relatively high dosages employed in animals. Thus, in the case of Myleran, some unchanged drug was excreted in rat urine.

In vitro studies of the metabolic alteration of HN2 and TEM showed this to be enzymatic in nature.

References

Alpert, L. K. & M. K. McCabe. 1957. Studies with sulfur mustard in human neoplastic disease. Proc. Am. Assoc. Cancer Research. **2**: 184.

Axelrod, J. 1956. The enzymatic N-demethylation of narcotic drugs. J. Pharmacol. Exptl. Therap. **117**: 322–330.

Barron, E. S. G., G. R. Bartlett & Z. B. Miller. 1948. Effect of nitrogen mustards on enzymes and tissue metabolism. J. Exptl. Med. **87**: 489–519.

Boursnell, J. C., J. A. Cohen, M. Dixon, G. E. Francis, G. D. Greville, D. M. Needham & A. Wormall. 1946. Studies on mustard gas (β,β'-dichlorodiethyl sulphide) and some related compounds. V. The fate of injected mustard gas (containing radioactive sulphur) in the animal body. Biochem. J. **40**: 756–764.

Craig, A. W. & H. Jackson. 1955. The metabolism of ^{32}P-labeled triethylenephosphoramide in relation to its anti-tumour activity. Brit. J. Pharmacol. **10**: 321–325.

Davison, C., R. S. Rozman, L. Bliss and P. K. Smith. 1957. Studies on the metabolic fate of bis(2-chloroethyl)sulfide (mustard gas) in the mouse and human. Proc. Am. Assoc. Cancer Research. **2**: 195.

Goldenthal, E. I., M. V. Nadkarni & P. K. Smith. 1956. Excretion of radioactivity following administration of ethyleneimino-labeled TEM-C^{14} in normal mice. Proc. Am. Assoc. Cancer Research. **2**: 110.

Maller, R. K. & C. Heidelberger. 1956. Further studies on the metabolism of N-(3-oxapentamethylene)-C^{14}-N,N″-diethylenethiophosphoramide (OPSPA) in the rat and human. Proc. Am. Assoc. Cancer Research. **2**: 130.

Nadkarni, M. V., E. I. Goldenthal & P. K. Smith. 1953. Tissue distribution and excretion of N,N′,N″-triethylenephosphoramide P^{32} and 2,4,6 triethyleneimino s-triazine-C^{14} in normal and tumor-bearing mice. Proc. Am. Assoc. Cancer Research. **1**: 39.

Nadkarni, M. V., E. I. Goldenthal & P. K. Smith. 1954. The distribution of radioactivity following administration of triethylenimino s triazine C^{14} in tumor bearing and control mice. Cancer Research. **14**: 559–562.

Nadkarni, M. V., E. I. Goldenthal & P. K. Smith. 1957a. The distribution of radioactivity following administration of triethylenephosphoramide P^{32} in tumor bearing and control mice. Cancer Research. **17**: 97–101.

Nadkarni, M. V., E. G. Trams & P. K. Smith. 1956. Observations on the rapid disappearance of radioactivity from blood after intravenous triethylene melamine C^{14}. Proc. Am. Assoc. Cancer Research. **2**: 136.

Nadkarni, M. V., E. G. Trams & P. K. Smith. 1957b Studies with isotopically labeled triethylene melamine, triethylenephosphoramide and 1:4 dimethane sulfonoxybutane in cancer patients. Proc. Am. Assoc. Cancer Research. **2**: 235.

Philips, F. S. 1950. Recent contributions to the pharmacology of bis(2-haloethyl)-amines and sulfides. Pharmacol. Revs. **2:** 281–323.

Skipper, H. E. 1953. A review: on the mechanism of action of certain temporary anti-cancer agents. Cancer Research. **13:** 545–551.

Skipper, H. E., L. L. Bennett & W. H. Langham. 1951. Over-all tracer studies with C^{14}-labeled nitrogen mustard in normal and leukemic mice. Cancer. **4:** 1025–1027.

Trams, E. G. & C. T. Klopp. 1955. Distribution of radiocarbon in liver choline, creatine, and methionine after the injection of methyl (C^{14} bis-(β-chloroethyl)amine. Cancer Research. **15:** 617–619.

Trams, E. G. & M. V. Nadkarni. 1956. Studies on the N-dealkylation of nitrogen mustard and triethylene melamine by liver homogenates. Cancer Research **16:** 1069–1075.

Trams, E. G., R. A. Salvador, G. Maengwyn-Davies & V. De Quattro. 1957. Studies on the uptake, distribution and metabolic fate of 1:4 dimethanesulfonoxybutane (Myleran) in the rat. Proc. Am. Assoc. Cancer Research. **2:** 256.

Wheeler, G. P., J. S. Morrow & H. E. Skipper. 1955. Studies with mustards. II. Paper chromatography and radioautography of reaction mixtures of various compounds with S-35-sulfur mustard. Arch. Biochem. Biophys. **57:** 133–139.

DISCUSSION

Charles Heidelberger and R. K. Maller (*McArdle Memorial Laboratory, University of Wisconsin, Madison, Wis.*): I propose to discuss some work we have done in which results have differed from those reported by Paul K. Smith and his colleagues. We have been studying the metabolism of *N*-(3-oxapentamethylene)-C^{14}-*N′*,*N″*-diethylenethiophosphoramide (OPSPA) labeled also with P^{32}. This compound has been very potent in its activity against certain transplanted tumors and has caused complete regression of established Flexner-Jobling carcinomas following a single, nontoxic injection.[1]

In studies on tissue distribution we found, as did Smith and his associates, that in rats and human cancer patients there was no selective localization of radioactivity in any tissue, including the tumor. Studies directed toward determining *in vivo* the mechanism of the tumor-inhibiting action of this drug thus far have failed completely. However, some results on metabolic transformations of OPSPA have been obtained; I believe these are of some interest, particularly in connection with Smith's presentation.

We have shown by suitable carrier crystallization and chromatographic procedures that OPSPA is metabolized in rats and humans to morpholine, inorganic phosphate and, chiefly, to *N*-(3-oxapentamethylene)-*N′*,*N″*-diethylenephosphoramide (MEPA), the desulfurized product (FIGURE 1).[2]

The data in TABLE 1 show that in rat urine, in Flexner-Jobling carcinoma aqueous-soluble fraction, and in human plasma, the total radioactivity was accounted for as these three metabolites. However, in human urines there was a very considerable amount of radioactivity as yet uncharacterized.

Since MEPA was present in appreciable amounts in blood, tissues, and urine, while OPSPA was not, it appeared likely that the tumor inhibition observed was due to metabolically produced MEPA, rather than to the administered OPSPA. Tests of both drugs on established Flexner-Jobling carcinomas in the same experiment showed them to be of equal potency, which lends support to the metabolic findings.

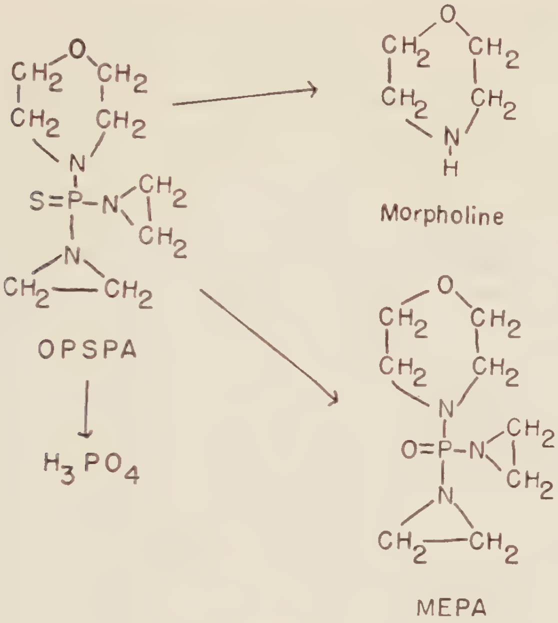

FIGURE 1

TABLE 1
PER CENT OF TOTAL C^{14} IN SAMPLES

	Morpholine	MEPA	OPSPA	Total
Rat, intraperitoneal				
Urine, 2 hours	2.8	84.0	1.7	88.5
Urine, 24 hours	22.1	65.8	0.9	88.8
Liver, aq. sol., 2 hours	20.8	28.2	0.0	49.0
Tumor, aq. sol., 2 hours	33.5	58.5	1.1	93.1
Human, intravenous				
Urine, 8 hours	0.3	28.4	3.2	31.9
Urine, 24 hours	1.8	25.0	2.6	29.4
Plasma, 1 hour	10.4	72.4	21.2	104.0
Plasma, 12 hours	32.5	55.2	29.7	117.0
Human, intratumoral				
Urine, 8 hours	0.6	39.1	—	39.7
Urine, 24 hours	1.5	44.9	—	46.4
Human, intramuscular				
Urine, 8 hours	—	23.0	1.1	24.1
Urine, 24 hours	6.7	13.8	0.9	21.4

In conclusion, I emphasize the fact that ethylenimine phosphoramides, as illustrated here, have a considerably greater metabolic stability than the other classes of alkylating agents reported by Smith, since an active tumor-inhibiting metabolite has been shown to be present in the circulating blood, in tissues, and in urine.

References

1. Heidelberger, C. & M. E. Baumann. 1957. Studies on OPSPA. I. The effect of several phosphoramides on transplanted tumors. Cancer Research. **17**: 277–283.
2. Maller, R. K. & C. Heidelberger. 1957. Studies on OPSPA. II. Distribution and excretion of radioactivity following administration of OPSPA-C^{14} and OPSPA-P^{32} to the rat. Cancer Research. **17**: 284–290.

TUMOR-INHIBITORY EFFECTS OF ALKYLATING AGENTS

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Although earlier studies had been conducted with sulfur mustard against both experimental and clinical cancer,[1] it was not until experiments were made during World War II on the pharmacological and pathological effects of the nitrogen mustards that there was real encouragement in the practical use of alkylating agents for the treatment of malignant disease. It was probably the dramatic results of the early studies[2] and the experience accumulated in an organized study[3-6] that provided much of the basis for the expansion in cancer chemotherapy studies after the war. The tumor inhibitory activities of a number of alkylating agents in man and or experimental animals are well established.[7-11] These include the β-chloroethyl amines, ethylenimines, sulfonoxy alkanes, epoxides, and triazenes.

Additional new agents were reported at the conference on which this monograph is based[12-16] and at the subsequent meeting of the American Association for Cancer Research.[17-25] Thus, it is apparent that some investigators, whether with new approaches[26-28] or with the conviction that further variations of the theme may provide a more useful biological activity, will continue to bring forward new alkylating agents as candidates for clinical trial. This will repeatedly raise the question of whether the new compounds are sufficiently superior to those already available to warrant the use of the relatively limited possibilities for clinical trial of experimental agents in cancer. Studies in biochemistry and pharmacology may be expected to decrease the number that eventually reach clinical study, but it remains an obligation of the experimental cancer chemotherapists to minimize the demands upon the pharmacologists and upon clinical resources. It will be the purpose of this paper to review the information gained from experimental animal studies and to consider how the obligations may be met.

In the light of the pharmacological properties of the nitrogen mustards[29, 30] and of the early results in animal and human studies,[2] it was natural that studies in our laboratories be initiated with mouse leukemia. Burchenal *et al.*[31, 32] reported on over fifty nitrogen mustards tested against several lines of leukemia in AK mice. In these studies no compound better than HN2 was noted, although a few mustards were of about the same degree of effectiveness in prolonging the survival time of the treated mice. These reports were made either in terms of effect relative to the standard (HN2) or at least with HN2 run as a positive control in each set of experiments. The importance of this should be obvious in the consideration of new candidates. It was noted that compounds with only one β-chloroethylamine group were ineffective. It was observed further that effectiveness could be retained when the β-chloroethyl groups in HN2 were replaced by either β-bromoethyl or β-chloropropyl groups. It should be noted, however, that

Peczenik[33] has observed inactivity of the bromo compounds. The activities of the bromoethyl aryl amines and of the β-chloropropyl amines were observed by Haddow *et al.*[34] with the Walker carcinosarcoma 256. The latter group of investigators also emphasized the need for two reactive groups, and the data later led to the hypothesis that alkylating agents were effective because of their ability to crosslink chromatids.[35, 36] Other evidence could be brought forward, however, that this interesting possibility is not essential as a mechanism to explain the effects observed in chromosomes and indeed in experimental cancer.[37] Recently, interest in the "one-armed" alkylating agents has been revived by published reports of the activity of ethyl methanesulfonate[38] and of hemimustard.[15] Several monoethylenimines have been active in tests against sarcoma 180, but the effect was small.[39]

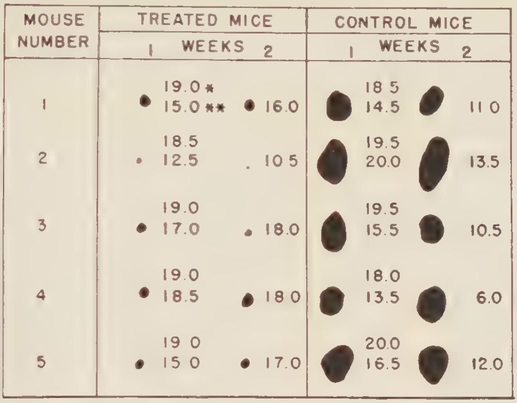
A cytocidal test in mouse leukemia developed in early studies by Burchenal *et al.*[40] is capable of revealing the amount of an effective agent that must be injected into the mouse to sterilize it of added leukemia cells. Although a comparatively small number of mustards and other compounds were reported in these experiments, the procedure should provide a method of testing for other more effective agents to be used in conjunction with surgery.[41]

The Chester Beatty Institute is well known for its most active and effective program on alkylating agents. The studies of Haddow and his associates, using chiefly the Walker carcinoma 256, have led to the development of such materials as the aromatic mustard, R48, *N*,*N*-di(2-chloroethyl)aniline;[41] CB 1348, *p*-(*N*,*N*-di-2-chloroethyl)amino phenylbutyric acid;[42] CB 3025, *p*-(*N*,*N*-di-2-chloroethyl)amino-L-phenylalanine;[43] Myleran, 1,4-bis(methanesulfonoxy)butane;[44, 45] and very likely others yet to be disclosed. As will be mentioned later, Sugiura[25, 46-49] has found the Walker carcinosarcoma 256 among tumors useful in detecting active alkylating agents.

Another investigative group (Hendry, Walpole, and their associates, at Imperial Chemical Industries Limited, Manchester, England) has utilized the Walker 256 to investigate ethylenimines,[50] epoxides,[51] and miscellaneous compounds.[52, 53] This was one of three laboratories[54-57] that more or less simultaneously found 2,4,6-trisethylenimino-*s*-triazine (triethylene melamine, TEM) effective in experimental cancer.

Because of the usefulness of mouse leukemia for the detection of effective mustards, our standard screening program utilizing the Crocker mouse sarcoma 180 was devoted for a considerable period to tests of other materials. It was actively used later to investigate a number of ethylenimines, beginning with TEM[39] and including a number of phosphoramides.[58] Mustards also were studied when possible, and the experiments led to a demonstration of the superiority of SK 1424, 3-bis(β-chloroethyl)aminomethyl-4-methoxymethyl-5-hydroxy-6-methylpyridine over HN2[60] (TABLE 1). The experiments on the phosphoramides tested against sarcoma 180[58] have been extended with others that subsequently became available, but by this test none of the newer ones has appeared to be better than the best of the earlier ones, DEPA (phosphoric acid diethylamide diethylenimide), TEPA (*N*,*N*′,*N*″-triethylene phosphoramide), and TSPA (*N*,*N*′,*N*″-triethylene thiophosphoramide, Thio-TEPA).[59] Typical of the results observed on one of the tumors with one of these agents is the retardation of growth of sarcoma 180 shown in area diagrams in FIGURE 1.

In our screening tests with sarcoma 180, Myleran revealed no tumor inhibition, but there was interest in testing homologues of this compound that



* WEIGHT OF ANIMAL AT START OF THERAPY.

** WEIGHT OF ANIMAL AT TIME OF TUMOR MEASUREMENT.

FIGURE 1. Inhibition of sarcoma 180 by phosphoric acid diethylamide diethylenimide (DEPA).

TABLE 1
INHIBITION OF SARCOMA 180 BY SK 1424 AND HN2

Compound	Mg./kg./day	Groups of 5 mice	Graded results, No. of groups showing			Toxicity, percentage of deaths	
			+	±	−	7 days	28 days
HN2	0.8	12	0	8	4	5	26
	1.0	5	0	2	3	16	24
	1.4	5	2	2	1	12	68
SK 1424	2.5	4	0	2	2	0	0
	5.0	5	0	5	0	8	32
	7.5	9	4	5	0	9	20
	10.0	12	9	3	0	4	45
	15.0	10	10	0	0	6	48

had been reported by the Chester Beatty group.[15] Some of these showed only a slight activity at tolerated doses, but as the test tumor, sarcoma 180, was not responding particularly well to HN2, it was decided to test one of the homologues of Myleran in a tumor spectrum containing tumors more susceptible than sarcoma 180 with respect to the alkylating agents.[16-18]

Studies in the tumor spectrum have been conducted in a fashion very similar to those for the sarcoma 180 screening program. The procedure has been described before[16-18] and, briefly, is as follows: (1) the mice or rats are weighed; (2) the tumor pieces (1 to 2 mm.) are transplanted subcutaneously by trocar into the axillary region; (3) intraperitoneal injections of the test material are started 24 hours later and continued once daily for 1 week; (4) the hosts are weighed at the end of the injection period and the tumors measured in two dimensions with calipers on the eighth post-transplant day; (5) the measurements are repeated at weekly intervals. A few differences from the sarcoma 180 screening procedure, such as frequency of injections and elimination of one day's injections over the week ends in the spectrum tests, may be the basis for some differences in degrees of effectiveness in the results obtained with sarcoma 180 in the two programs.

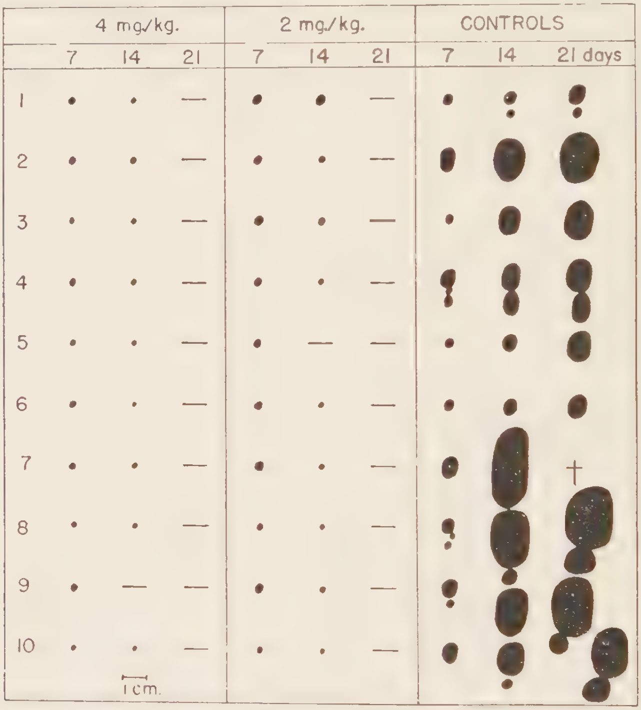


FIGURE 2. Effect of N,N',N'' triethylene thiophosphoramide (TSPA, Thio-TEPA) on carcinoma 1025 in mice.

Through the spectrum studies it has been found that the phosphoramides are quite effective against a number of tumors: the Walker carcinosarcoma 256, the Jensen rat sarcoma, the Flexner-Jobling carcinoma, and rat sarcoma 39; and, in the mouse, carcinoma 1025, the Wagner and Ridgway osteogenic sarcomas, mammary adenocarcinoma E 0771, the Gardner lymphosarcoma, the Miyono adenocarcinoma, sarcoma 180, and various ascites tumors.[48] These include the tumors affected by HN2 and the mustard 3-bis(β-chloroethyl)aminomethyl-4-methoxymethyl-5-hydroxy-6-methylpyridine dihydrochloride (SK 1424).[16] In FIGURE 2 may be seen the effect of N,N',N''-triethylene thiophosphoramide on mouse carcinoma 1025. A comparison of the effects of DEPA, TEPA, and TSPA titrated in the tumor spectrum indicates that, for doses comparable in toxic effects, TEPA and TSPA are superior to DEPA. The comparison is summarized in TABLE 2. For the detailed data from which the table was prepared, reference should be made to the original publication.[18] In a similar way Myleran and its nonane homologue were compared. TABLE 3 presents a summary based upon comparisons at levels of comparable toxicity selected from titrations. Detailed data are in press.[19] This table indicates a superiority of the nonane homologue over Myleran as far as this group of experimental animal tumors is concerned. FIGURE 3 presents area diagrams showing the effect that can be observed against a rat tumor with the nonane homologue.

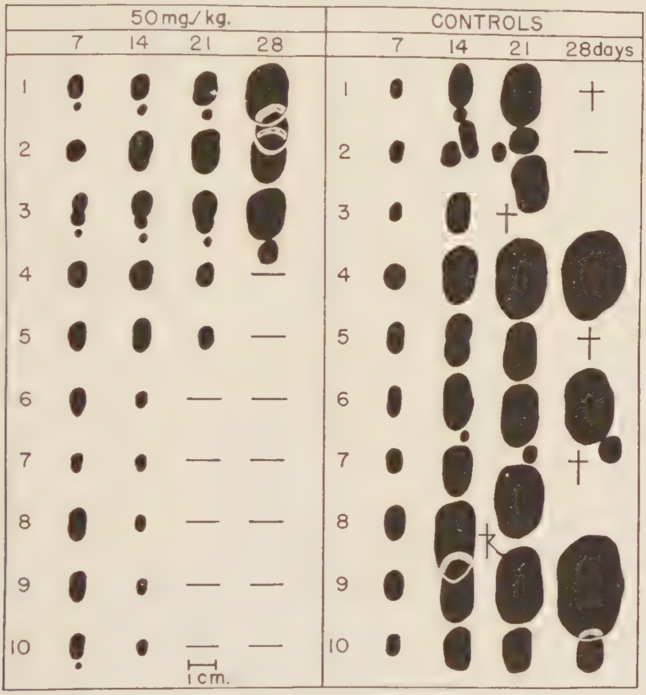


FIGURE 3. Effect of 1,9-bis(methanesulfonoxy)nonane (DMSN, Nonane) on Flexner-Jobling carcinoma in rats.

The question frequently arises concerning the significance of results obtained with transplantable tumors when treatment is initiated a day after implantation of the tumor. It has been considered that tumors would be more susceptible to retardation of growth under such conditions. Our data, as well as those of many others, support this view; however, agents detected

TABLE 2

COMPARISON OF PHOSPHORAMIDES FOR RETARDATION OF GROWTH OF MOUSE AND RAT TUMORS*

Compound	Mouse tumors†												Rat tumors†			
	Ehr	C 1025	GE	ROS	GLS	HP	S 180	T 241	Ma 387	E 0771	Ca 63	MC	W 256	FJC	JRS	MSL
TEPA‡...	+*	+++	+	++	++	±	+	−	±	+	±	++	+++	+++	+++	−
TSPA§...	+	+++	+	+++	++ to +++	±	+	±	+	+	++	+	++ to +++	+++	+++	±
DEPA‖...	−	++	−	++	−	±	±	−	±	−	±	−	++ to +++	+++	+++	−

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* The tumor growth retardation has been graded as follows:

−	Growth more than $\frac{3}{4}$ the average diameter of the controls.
±	Growth from $\frac{1}{2}$ to $\frac{3}{4}$ the average diameter of the controls.
+	Growth from $\frac{1}{4}$ to $\frac{1}{2}$ the average diameter of the controls.
++	No growth or growth to $\frac{1}{4}$ average diameter of the controls.
+++	Destruction of tumor.

† The tumors have been abbreviated as follows: Ehr, Ehrlich carcinoma; C 1025, carcinoma 1025; GE, Grand epidermoid; ROS, Ridgway osteogenic sarcoma; GLS, Gardner lymphosarcoma; HP, Harding Passey mouse melanoma; S 180, sarcoma 180; T 241, sarcoma T 241; Ma 387, sarcoma Ma 387; E 0771, mammary adenocarcinoma E 0771; Ca 63, Bashford carcinoma 63; MC, Miyono adenocarcinoma; W 256, Walker carcinosarcoma 256; FJC, Flexner-Jobling rat carcinoma; JRS, Jensen rat sarcoma; MSL, Murphy-Sturm rat lymphosarcoma.

‡ *N*,*N*′,*N*″-Triethylene phosphoramide.

§ *N*,*N*′,*N*″-Triethylene thiophosphoramide.

‖ Phosphoric acid diethylamide diethylenimide.

TABLE 3

COMPARISON OF DIMESYL ALKANES FOR TUMOR GROWTH RETARDATION

Compound	Mouse tumors												Rat tumors			
	Ehr	C 1025	GE	ROS	GLS	HP	S 180	T 241	Ma 387	E 0771	Ca 63	MC	W 256	FJC	JRS	MSL
DMSB*.......	±	+ to ++	—	—	—	±	—	—	—	+	—	—	++	+	++	—
DMSN†......	±	++	++	++	± to +	±	—‡	— to ±	—	+	+	+	++	+++	+++	— to ±

See footnotes to TABLE 2 for explanations of tumor abbreviations and grading of tumor growth retardation.

* 1,4-Di(methanesulfonoxy)butane; Myleran.

† 1,9-Di(methanesulfonoxy)nonane; Nonane.

‡ ± In the screening program study.

by this early treatment also have the ability to damage well-established tumors sufficiently to cause relatively high rates of tumor regression. TABLE 4 summarizes a few of the results obtained with some of the alkylating agents tested against the Flexner-Jobling carcinoma. Such tests are run more or less routinely for all tumors in our spectrum whenever good retardation in growth has been observed in early therapy tests. The data in TABLE 4 were obtained at different times spread over a number of years and therefore are not, with certainty, comparable. This results from the fact that our test

TABLE 4
RESULTS ON 7-DAY-OLD FLEXNER-JOBLING RAT CARCINOMA

Compound	Dose (mg./kg./day)	Deaths	Wt. change (gm.)	Percentage of regressions
HN2	0.5	1/10	—	40
HN2	0.25	0/10	—	40
SK 1424	5.0	2/10	—	70
TEM	0.25	1/30	—	93
TEM	0.125	0/20	—	95
DEPA	2.5	0/40	4/24	90
DEPA	1.0	0/30	14/23	67
TEPA	1.0	1/70	7/27	77
TSPA	2.0	0/50	−2/26	80
TSPA	1.0	0/20	13/18	75
TSPA	0.5	0/20	22/27	40
Myleran	5.0	0/10	15/20	—
Nonane	50.0	0/40	13/16	70

TABLE 5
COMPARISON OF TUMOR RETARDATIONS IN TWO LINES OF SARCOMA 180

Compound	Relative tumor diameters	
	SKI	SRI
Diaminobiuret	0.79	0.63
Sulfomerthiolate	0.78	0.48
6-Mercaptopurine	0.81	0.55
Netropsin	0.81	0.64
TEM, 0.75 mg	0.48	0.32
TEM, 0.50 mg	0.66	0.42
9, 10-Dimethyl-1,2-benzanthracene	0.80	0.58
Controls	1.00	1.00

SKI and SRI represent, respectively, the Sloan-Kettering Institute and Southern Research Institute lines of sarcoma 180. The response of the SRI line of sarcoma 180 is representative of that given a few years earlier by the SKI line. The SRI line was derived from the SKI line about three years prior to this comparison.

object has not necessarily remained invariable over the years. An example of a changed susceptibility of a line of sarcoma 180 has been reported.[61] Tests of some alkylating agents aided in revealing the decrease in sensitivity, as shown in TABLE 5.

Ascites tumor cells have been used extensively[62] to test for alkylating agents, for they have responded, as indicated by various reports.[19, 63, 64] This is discussed more extensively elsewhere in this publication by Creech.[65]

Discussion

In this paper we have tried to emphasize from our studies that there are a number of tumors quite responsive to alkylating agents and that these tumors should be useful in the detection and study of as yet unsynthesized agents with this type of action. Equally important and helpful for studies on the potential usefulness of compounds and for mechanism-of-effect studies may be some of the tumors thus far unaffected by the alkylating agents. Thus, a spectrum of tumors, as well as certain selected susceptible tumors, may provide useful information about new candidate alkylating agents. Of possible interest, also, may be those tumors considered to have become resistant to an alkylating agent.[66-68] The desirability and even the necessity of testing a new agent in a titration in parallel with an established standard, if one is seeking new and more effective agents based upon the earlier successes, have been stressed. In the literature may be found comparisons that lack the necessary data to justify them. In one report[69] it has been suggested that *N,N′*-4-(methyl-*m*-phenylene)-bis(aziridine carboxamide) is superior to TEM; however, the data presented are so inadequate as to cause surprise at the authors' conclusion. In another report[70] it has been suggested that the phosphoramides are superior to TEM, but this conclusion was based upon studies in which the phosphoramides were pushed to dose levels comparatively higher in terms of toxicity than those used for TEM.

Various reports of individual alkylating agents may be found in the literature. An attempt has been made to summarize in TABLE 6 the more generally established ones. No attempt has been made to compare the effectiveness among these compounds, for in most instances the data have not provided enough information for comparisons even in one tumor system. While it is believed that the most important references have been included for the selected alkylating agents, it is possible that there are important but unintentional omissions. Reference to the literature cited in the table will emphasize the need for more comparative studies. The usefulness of the inclusion of a standard agent in tests with new tumors or agents was again demonstrated in a recent report[71] in which tests of sarcolysin on tumors unknown to us could be better evaluated because the authors had given results with HN2 and R48 as a frame of reference.

Summary

A number of experimental animal tumors are useful for the study of alkylating agents and should be able to provide information on the possible superiority of new alkylating agents. In any comparison a titration of the

TABLE 6

SOME ANTITUMOR ACTIVITIES OF SELECTED ALKYLATING AGENTS

Tumor†	Compounds*											
	HN2	Nitromin	SK 1424	R48	CB 1348	CB 3025	TEM	TEPA	TSPA	DEPA	DMSB	DMSN
Ehrlich (A)	+(63)‡	+(63)	+(63)				+(63)	+(63)(48)	+(63)(48)	+(63)(48)	+(49)(63)	+(49)(63)
S 180 (A)	+(63)	+(63)	+(63)				+(63)	+(48)(63)	+(48)(63)	−(48)(63)	−(49) +(63)	−(49) +(63)
Krebs (A)	+(63)(78)	+(63)	+(63)(78)		+(76)		+(63)(78)	+(48)(63)	+(48)(63)	−(48)(63)	+(63) +(49)	+(49)(63)
Ehrlich	+(63)	−(63)	+(88)					+(48)	+(48)	−(48)	+(49)	+(49)
S 180	−(46) +(60)(75) +(78)	+(63)	+(78)		+(76)		+(39)(79) +(47)(78)	+(48)(58)	+(48)	+(48)(58)	−(49) +(79)	−(49)
Krebs 2	+(78)		+(78)				+(78)					
S T 241	−(46)	−(88)	−(88)				+(47)	−(48)	+(48)	−(48)	−(49)	+(49)
S Ma 387		−(88)	−(88)				−(47)	+(48)	+(48)	+(48)	−(49)	−(49)
E 0771	−(46) +(88)	−(88)	+(88)				+(47)	+(48)	+(48)	−(48)	+(49)	+(49)
Ca 63	−(46)	−(88)	+(88)				+(47)	+(48)	+(48)	−(48)	−(49)	+(49)
M Ca	+(88)	+(88)	+(88)					+(48)	+(48)	+(48)	−(49)	+(49)
Ca 1025	+(46)	+(88)	+(46)				+(47)	+(48)	+(48)	+(48)	+(49)	+(49)
Grand Ca	+(88)	−(88)						+(48)	+(48)	−(48)	+(49)	+(49)
WOS	+(46)	−(88)	+(46)				+(47)	+(48)	+(48)	+(48)	−(49)	+(49)
ROS	+(46)	+(88)	+(46)				+(47)	+(48)	+(48)	−(48)	+(49)	+(49)
PLS	−(46)	+(88)	+(46)				+(47)	+(48)	+(48)	−(48)	−(49)	+(49)
MLS	+(46)	−(88)	+(88)				+(47)	+(48)	+(48)	−(48)	−(49)	+(49)
GLS	+(88)	−(88)	−(88)				+(47)	+(48)	+(48)	−(48)	−(49)	+(49)
H. P. Mel	−(46)	−(88)	−(46)		+(88)	+(77)	−(47)	−(48)	+(48)	+(48)	+(49)	+(49)
Lewis BC	−(88)	−(88)	+(88)					+(48)	+(48)	−(48)	−(49)	+(49)
Lewis LC	−(88)	+(88)	+(88)					+(48)	+(48)	+(48)	−(49)	+(49)
S 37	+(33)			+(33)	+(76)		+(33)					
AK 4	+(89)		+(89)				+(56)(73)	+(89)		+(89)	+(89)	
AK 1394	+(31)	+(32)	+(89)	+(89)								
AK 9417	+(31)	+(89)	+(89)	+(89)								
Leukemia 82	+(89)				+(89)		+(89)	+(89)	+(89)		+(89)	
Leukemia 8174	+(89)				+(89)		+(89)	+(89)	+(89)		+(89)	

RC	−(78)		+(78)				−(78)					
C 3 H-BA	+(78)		+(78)				+(78)					
TD	−(78)		+(78)				+(78)					
TL	−(78)		+(78)				+(78)					
Ca 755	+(78)		+(78)				+(78)					
15091 a	+(78)		−(78)				+(78)					
RSM	+(71)			+(71)		+(71)						
W 256	+(33)(46)	+(88)	+(46)	+(33)(34)	+(76)	+(88)(76)	+(53)(47) +(50)(33) +(68)(69)	+(48)(68)	+(48)	+(48)	+(44)	+(49)
F. J. Ca	+(46)	+(88)	+(46)				+(47)(74)	+(48)	+(48)	+(48)	+(49)	+(49)
JRS	+(88)	+(88)	+(46)				+(47)	+(48)	+(48)	+(49)	+(49)	+(49)
R 39	+(46)	+(88)	+(46)				+(47)	+(48)	+(48)	+(48)		
MRLS	+(88)	+(88)					−(47)	−(48)	+(48)	−(49)	−(49)	+(49)
Yoshida (A)	+(85)(62)	+(62)(86)										
Shay							+(70)	+(70)	+(80)			
S 45	+(71)			+(71)		+(71)						
M-1	+(71)			+(71)		+(71)						
S 231							+(81)					

In addition to these data and others in the text, attention should be called to: 2,5-bis(ethylenimino)-1,4-benzoquinone active against Yoshida rat sarcoma solid *in vivo*, Ehrlich solid *in vitro*, and TEM, HN2 and the "quinone" against Yoshida and Ehrlich cells *in vitro*,[53] 2,5-bis(ethylenimino)hydroquinone active against S 180, Ehrlich carcinoma, adenocarcinoma E 0771, adenocarcinoma Ciba, Ciba osteogenic sarcoma, Walker 256, and the Flexner-Jobling carcinoma *in vivo*,[52] 1,2-bis(β-chloroethylthio)ethane active against Ehrlich (ascites and solid), C3H-SX (ascites and solid), 6C3HED (ascites and solid), C3HBA (solid), and HN2 active against all of these plus S 180 (solid) and AT 3 (solid);[54] 2,4-dichloro-6-ethyleniminopyrimidine, 4,6-dichloro-2-ethyleniminopyrimidine, and 4-chloro-2,6-bis(ethylenimino)pyrimidine against the Walker 256;[illegible] Dopan, 2,6-dihydroxy-4-methyl-5-di(2-chloroethyl)amino pyrimidine active against rat sarcomas 45 and M-1;[72] 2,4-bis(ethylenimino)-6-chloropyrimidine active against Walker carcinosarcoma 256, sarcoma 180, and nearly all of the rest of the spectrum tested.[illegible]

* Explanation of compound abbreviations: HN2 = methyl-bis(β-chloroethyl)amine; Nitromin = the oxide of HN2; SK 1424 = 3-bis(β-chloroethyl)aminomethyl-4-methoxymethyl-5-hydroxy-6-methylpyridine; R48 = N,N-di(2-chloroethyl)aniline; CB 1348 = p-(N,N-di-2-chloroethyl)aminophenylbutyric acid; CB 3025 = p-N,N-di-2-chloroethylamino-l-phenylalanine (also for convenience the data for sarcolysin are included here); TEM = 2,4,6-tris(ethylenimino)-s-triazine; TEPA = N,N′,N″-triethylene phosphoramide, TSPA = N,N′,N″-triethylene thiophosphoramide (Thio-TEPA); DEPA = phosphoric acid diethylamide diethylenimide; DMSB = Myleran = 1,4-bis(methanesulfonoxy)butane; DMSN = 1,9-bis(methanesulfonoxy)nonane.

† Explanation of tumor abbreviations: Ehrlich (A), S 180 (A), Krebs (A) are the ascitic forms of the Ehrlich carcinoma, of Crocker sarcoma 180, and of the Krebs 2 carcinoma, respectively; S T 241 = sarcoma T 241; S Ma 387 = sarcoma Ma 387; E 0771 = mammary adenocarcinoma E 0771; Ca 63 = carcinoma 63; M Ca = Miyono adenocarcinoma; Ca 1025 = carcinoma 1025; Grand Ca = Grand epidermoid carcinoma; WOS and ROS = Wagner and Ridgway osteogenic sarcomas, respectively; PLS, MLS, and GLS are the Patterson, Mecca, and Gardner lymphosarcomas, respectively; H. P. Mel = Harding-Passey melanoma; Lewis BC and LC = Lewis bladder and lung carcinomas, respectively, S 37 = sarcoma 37; AK 4, AK 1394, AK 9417, Lines 82 and 8174 = various mouse leukemias; RC, C 3 H-BA, TD, TL, Ca 755, 15091 a, and RSM = various mouse adenocarcinomas; W 256 = Walker 256, Walker carcinoma 256 and Walker carcinosarcoma 256; F. J. Ca = Flexner-Jobling rat carcinoma; JRS = Jensen rat sarcoma; R 39 = rat sarcoma 39; MRLS = Murphy rat lymphosarcoma; Yoshida (A) = Yoshida rat ascites tumor; Shay = Shay rat chloroleukemia; S 45 = rat sarcoma 45; M-1 = M-1 rat spindle cell sarcoma; S 231 = sarcoma 231.

‡ Numbers in parentheses designate bibliographical references.

compound for effective dose range should be made along with a standard effective compound for evaluation of the two materials with respect to their toxic doses. Use of more than one type of tumor should aid considerably in the definition of the potential usefulness of the compounds. Comparisons among all of the better agents are not yet adequate as far as the experimental animal tumor data are concerned. Our studies indicate the superiority of SK 1424 over HN2, of TEPA and Thio-TEPA over DEPA, and of the nonane homologue of Myleran over the latter. Data are inadequate to rank these compounds further and we require added definition of the relative utility of these compounds in the clinic in order to select the most appropriate criteria of animal tumor evaluation.

References

1. Adair, F. E. & H. J. Bagg. 1931. Experimental and clinical studies on the treatment of cancer by dichlorethylsulphide (mustard gas). Ann. Surg. **93**: 190–199.
2. Goodman, L. S. & A. Gilman. 1955. *In* The Pharmacological Basis of Therapeutics. 2nd ed. : 1415. Macmillan. New York, N. Y.
3. Rhoads, C. P. 1946. Nitrogen mustards in the treatment of neoplastic disease. J. Am. Med. Assoc. **131**: 656–658.
4. Goodman, L. S., M. M. Wintrobe, W. Dameshek, M. J. Goodman, A. Gilman & M. T. McLennan. 1946. Nitrogen mustard therapy. J. Am. Med. Assoc. **132**: 126–132.
5. Jacobson, L. O., C. L. Spurr, E. S. G. Barron, T. Smith, C. Lushbaugh & G. F. Dick. 1946. Nitrogen mustard therapy. J. Am. Med. Assoc. **132**: 263–271.
6. Karnofsky, D. A., L. F. Craver, C. P. Rhoads & J. C. Abels. 1947. An evaluation of methyl-bis(β-chloroethyl)amine hydrochloride and tris(β-chloroethyl)amine hydrochloride (nitrogen mustards) in the treatment of lymphomas, leukemia and allied diseases. *In* Approaches to Tumor Chemotherapy. : 319–337. F. R. Moulton, Ed. Am. Assoc. Advance. Sci. Washington, D. C.
7. Farber, S., R. Toch, E. M. Sears & D. Pinkel. 1956. Advances in chemotherapy of man. *In* Advances in Cancer Research. **4**: 1–17. J. P. Greenstein & A. Haddow, Eds.
8. U. S. Armed Forces Medical Library. Reference division. Bibliography section. 1956. Cancer chemotherapy. A bibliography of agents 1946–1954. Cancer Research. **16**(4): 267.
9. Stock, . C. C. 1954. Experimental cancer chemotherapy. Advances in Cancer Research. **2**: 425–492. J. P. Greenstein & A. Haddow, Eds.
10. Wilkinson, J. F. & F. Fletcher. 1947. Effect of β-chloroethyl amine hydrochlorides in leukemia, Hodgkin's disease and polycythemia vera. Lancet. **253**: 540–545.
11. Boyland, E., J. W. Clegg, P. C. Koller, E. Rhoden & O. H. Warwick. 1948. The effects of chloroethylamines on tumors, with special reference to bronchogenic carcinoma. Brit. J. Cancer. **2**: 17–29.
12. Elson, L. A. 1958. Hematological effects of the alkylating agents. Ann. N. Y. Acad. Sci. **68**(3): 826.
13. Jones, R., Jr., U. Jonsson, M. Browning, H. Lessner, C. C. Price & A. K. Sen. 1958. Initial clinical trial of the nitrogen mustard analogues of chloroquine and quinacrine. A preliminary report. Ann. N. Y. Acad. Sci. **68**(3): 1133.
14. Petrakis, N. L., D. A. Wood, S. R. Mettier, A. V. Constantini & S. M. Farber. 1958. Preliminary clinical evaluation of 1,2 bis(β chloroethylthio)ethane (SM-1) in patients with advanced lymphomas and neoplastic diseases. Ann. N. Y. Acad. Sci. **68**(3): 1151.
15. Seligman, A. M. & A. M. Rutenburg. 1958. Palliation of carcinomatosis with ascites by means of hemisulfur mustard. Ann. N. Y. Acad. Sci. **68**(3): 1181.

16. Papac, R., D. A. G. Galton, M. Till & E. Wiltshaw. 1958. Preliminary clinical trial of *p*-di(2-chloroethyl)amino-L-phenylalanine (CB 3025, Melphalan) and of di(2-chloroethyl) methanesulfonate (CB 1506). Ann. N. Y. Acad. Sci. **68**(3) : 1126.
17. Alpert, L. K. & M. M. McCabe. 1957. Studies with sulfur mustard in human neoplastic disease. Proc. Am. Assoc. Cancer Research. **2**: 184.
18. Bierman, H. R., A. G. Knudson, Jr., K. H. Kelly & G. M. Timmis. 1957. The influence of CB 2348 (dimethylmyleran) upon the hematopoietic system of leukemic and nonleukemic man. Proc. Am. Assoc. Cancer Research. **2**: 188.
19. Creech, H. J., R. F. Hankwitz, Jr., R. M. Peck & R. K. Preston. 1957. Comparative activities of a variety of nitrogen mustard analogs against ascites tumors in mice. Proc. Am. Assoc. Cancer Research. **2**: 195.
20. Hirschberg, E., A. Gellhorn & W. S. Gump. 1957. Laboratory evaluation of a new nitrogen mustard derivative, 2-[di-(2-chloroethyl)aminomethyl]benzimidazole. Proc. Am. Assoc. Cancer Research. **2**: 213.
21. Jones, R., Jr., U. Jonsson, H. Lessner, J. Graff & M. Browning. 1957. The effect of the nitrogen mustards related to chloroquine and quinacrine in patients with malignant disease. Proc. Am. Assoc. Cancer Research. **2**: 219.
22. Miller, J. I., S. P. Kramer, S. D. Gaby, C. Williamson, A. M. Seligman & B. Witten. 1957. Proc. Am. Assoc. Cancer Research. **2**: 232.
23. Petrakis, N. L., D. A. Wood, S. R. Mettier & A. V. Constantini. 1957. Preliminary clinical evaluation of 1,2-bis(β-chloroethylthio)ethane in patients with far advanced lymphoma and neoplastic diseases. Proc. Am. Assoc. Cancer Research. **2**: 238.
24. Rutman, R. J., C. F. Weaver & C. C. Price. 1957. Influence of the route of administration on the action of N-mustards. Proc. Am. Assoc. Cancer Research. **2**: 245.
25. Sugiura, K. 1957. The effect of triazenes on the growth of a variety of mouse, rat and hamster tumors. Proc. Am. Assoc. Cancer Research. **2**: 254.
26. Friedman, O. M. & A. M. Seligman. 1954. Preparation of N-phosphorylated derivatives of bis-beta-chloroethylamine. J. Am. Chem. Soc. **76**: 655–658.
27. Bergel, F., J. L. Everett, J. J. Roberts & W. C. J. Ross. 1954. Aromatic mustards possessing latent activity. Brit. Empire Cancer Campaign, 32nd Ann. Rept. : 35–36.
28. Hebborn, P. & J. F. Danielli. 1956. Selectivity of enzyme-activated nitrogen mustards. Nature. **177**: 25.
29. Gilman, A. & F. S. Philips. 1946. The biological actions and therapeutic applications of the β-chloroethyl amines and sulfides. Science. **103**: 409–415.
30. Philips, F. S. 1950. Recent contributions to the pharmacology of bis(2-haloethyl) amines and sulfides. Pharmacol. Revs. **2**: 281–323.
31. Burchenal, J. H., R. A. Lester, J. B. Riley & C. P. Rhoads. 1948. I. Effect of certain nitrogen mustards and carbamates on transmitted mouse leukemia. Cancer. **1**: 399–412.
32. Burchenal, J. H., J. R. Burchenal & S. F. Johnston. 1951. Chemotherapy of leukemia. III. Further studies on the effects of nitrogen mustards and related compounds on transmitted mouse leukemia. Cancer. **4**: 353–356.
33. Peczenik, O. 1952. Inhibition of tumor growth after oral and parenteral treatment with nitrogen mustards and triethylene melamine. Brit. J. Cancer. **6**: 262–269.
34. Haddow, A., G. A. R. Kon & W. C. J. Ross. 1948. Effects upon tumors of various haloalkylaryl amines. Nature. **162**: 824.
35. Goldacre, R. J., A. Loveless & W. C. J. Ross. 1949. Mode of production of chromosome abnormalities by the nitrogen mustards. Nature. **163**: 667–669.
36. Loveless, A. & S. Revell. 1949. New evidence on the mode of action of 'mitotic poisons.' Nature. **164**: 938–944.
37. Biesele, J. J., F. S. Philips, J. B. Thiersch, J. H. Burchenal, S. M. Buckley & C. C. Stock. 1950. Chromosome alteration and tumor inhibition by nitrogen mustards: the hypothesis of cross-linking alkylation. Nature. **166**: 1112–1113.
38. Haddow, A. & W. C. J. Ross. 1956. Tumor growth-inhibitory alkyl sulphonates. Nature. **177**: 995–996.

39. Buckley, S. M., C. C. Stock, M. L. Crossley & C. P. Rhoads. 1952. Inhibition of the Crocker mouse sarcoma 180 by certain ethylenimine derivatives and related compounds. Cancer. **5**: 144–152.

40. Burchenal, J. H., M. A. Cremer, B. S. Williams & R. A. Armstrong. 1951. Sterilization of leukemic cells *in vivo* and *in vitro*. Cancer Research. **11**: 700–705.

41. Economou, S. G., R. G. Mrazek, G. O. McDonald, D. P. Slaughter & W. H. Cole. 1958. The intraperitoneal use of nitrogen mustard at the time of operation for cancer. Ann. N. Y. Acad. Sci. **68** (3): 1097.

42. Ross, W. C. J., W. Davis, J. J. Roberts & J. L. Everett. 1952. New cytotoxic and radiomimetic agents. Brit. Empire Cancer Campaign Ann. Rept. **30**: 25–26.

43. Bergel, F. & J. A. Stock. 1954. Cyto-active amino-acid and peptide derivatives. Part I. Substituted phenylalanines. J. Chem. Soc. **2**: 2409–2417.

44. Haddow, A. & G. M. Timmis. 1951. Bifunctional sulfonic acid esters with radiomimetic activity. Acta Unio Intern. contra Cancrum. **7**: 469–471.

45. Haddow, A. & G. M. Timmis. 1953. Myleran in chronic myeloid leukemia. Chemical constitution and biological action. Lancet. **264**: 207–208.

46. Sugiura, K. & C. C. Stock. 1952. Studies in a tumor spectrum. I. Comparison of the action of methyl bis(2-chloroethyl)amine and 3-bis(2-chloroethyl)aminomethyl-4-methoxymethyl-5-hydroxy-6-methylpyridine on the growth of a variety of mouse and rat tumors. Cancer. **5**: 382–402.

47. Sugiura, K. & C. C. Stock. 1952. Studies in a tumor spectrum. II. The effect of 2,4,6-triethylenimino-s-triazine on the growth of a variety of mouse and rat tumors. Cancer. **5**: 979–991.

48. Sugiura, K. & C. C. Stock. 1955. Studies in a tumor spectrum. III. The effect of phosphoramides on the growth of a variety of mouse and rat tumors. Cancer Research. **15**: 38–51.

49. Sugiura, K., C. C. Stock & M. M. Sugiura. 1957. Studies in a tumor spectrum. V. The effect of esters of sulfonic acids on the growth of a variety of mouse and rat tumors. Cancer. **10**: 596–605.

50. Hendry, J. A., R. F. Homer, F. L. Rose & A. L. Walpole. 1951. Cytotoxic agents. III. Derivatives of ethyleneimine. Brit. J. Pharmacol. **6**: 357–410.

51. Hendry, J. A., R. F. Homer, F. L. Rose & A. L. Walpole. 1951. Cytotoxic agents. II. Bis-epoxides and related compounds. Brit. J. Pharmacol. **6**: 235–255.

52. Hendry, J. A., F. L. Rose & A. L. Walpole. 1951. Cytotoxic agents. I. Methylolamides with tumor inhibitory activity and related inactive compounds. Brit. J. Pharmacol. **6**: 201–234.

53. Walpole, A. L. 1951. The Walker carcinoma 256 in the screening of tumor inhibitors. Brit. J. Pharmacol. **6**: 135–143.

54. Hendry, J. A., F. L. Rose & A. L. Walpole. 1950. New cytotoxic agents with tumour inhibitory activity. IV. Theoretical speculations. Acta Unio Intern. contra Cancrum. **7**: 490–494.

55. Lewis, M. R. & M. L. Crossley. 1950. Retardation of tumor growth in mice by oral administration of ethylenimine derivatives. Arch. Biochem. **26**: 319–320.

56. Burchenal, J. H., M. L. Crossley, C. C. Stock & C. P. Rhoads. 1950. The action of certain ethylenimine (aziridine) derivatives on mouse leukemia. Arch. Biochem. **26**: 321–323.

57. Stock, C. & S. M. Buckley. 1951. Studies on the inhibition of sarcoma 180 in mice. Acta Unio Intern. contra Cancrum. **7**: 523–529.

58. Buckley, S. M., C. C. Stock, R. P. Parker, M. L. Crossley, E. Kuh & D. R. Seeger. 1951. Inhibition studies on some phosphoramides against sarcoma 180. Proc. Soc. Exptl. Biol. Med. **78**: 299–305.

59. Clarke, D. A. & C. C. Stock. Unpublished data.

60. Stock, C. C., S. Buckley, K. Sugiura & C. P. Rhoads. 1951. A comparison of the retardation of sarcoma 180 by SK 1424, 3-bis(β-chloroethyl)aminomethyl-4-methoxymethyl-5-hydroxy-6-methylpyridine, with that by HN_2, methyl bis(β-chloroethyl)amine. Cancer Research. **11**: 432–436.

61. Stock, C. C. 1955. Studies in experimental cancer chemotherapy. Current Res. in Cancer Chemotherapy. Rept. No. 3-55: 3–23.

62. Nakahara, W. 1952. *Addendum to* T. Yoshida, Studies on an ascites (reticulo-endothelial cell) sarcoma of the rat. J. Natl. Cancer Inst. **12**: 947–962.

63. Sugiura, K. 1956. Merits of ascites tumors for chemotherapeutic screening. Part I. Ann. N. Y. Acad. Sci. **63**(5): 962–973.

64. Creech, H. J. 1956. Merits of ascites tumors for chemotherapeutic screening. Part II. Ann. N. Y. Acad. Sci. **63**(5): 973–976.

65. Creech, H. J. 1958. Discussion: Part II. Biological effects of alkylating agents. Ann. N. Y. Acad. Sci. **68**(3): 868.

66. Hirono, I. 1955. Some properties of Yoshida sarcoma cells resistant to methyl bis(β-chloroethyl)amine-N-oxide. Proc. Soc. Exptl. Biol. Med. **88**: 147–149.

67. Crossley, M. L., J. B. Allison, D. R. Seeger & E. Kuh. 1957. IV. Chemotherapy of tumors in rats with piperazine-1,4-bis(N,N′-diethylene-phosphondiamide). Proc. Soc. Exptl. Biol. Med. **94**: 388–391.

68. Jackson, H. 1954. The development of resistance in the Walker carcinosarcoma to the action of triethylene melamine. Brit. J. Cancer. **8**: 336–345.

69. Herbut, P. A. & W. H. Kraemer. 1955. The effect of N,N′-(4-dimethyl-m-phenylene)-bis(aziridine carboxamide)(ESK 230) on Walker mammary carcinoma 256. Cancer Research. **15**: 614–616.

70. Sparks, S. J., M. L. Stevens, M. J. Landes, S. L. Halliday, D. McKenzie & J. H. Williams. 1953. The effect of a series of ethylenimine derivatives on myeloid chloroleukemia in the rat. Blood. **8**: 655–659.

71. Larionov, L. F., E. N. Shkodinskaja, V. I. Troosheikina, A. S. Khokhlov, O. S. Vasina & M. A. Novikova. 1955. Studies on the anti-tumor activity of p-di(2-chloroethyl)amino-phenylalanine (sarcolysine). 1955. Lancet. **269**: 169–171.

72. Larionov, L. F. 1956. Some biological and clinical results from the investigations of chloroethyl amines as anti-tumour drugs. Brit. J. Cancer. **10**: 26–32.

73. Burchenal, J. H., S. F. Johnston, M. A. Cremer, L. F. Webber & C. C. Stock. 1950. Chemotherapy of leukemia. V. Effects of 2,4,6-triethylene-imino-s-triazine and related compounds on transplanted mouse leukemia. Proc. Soc. Exptl. Biol. Med. **74**: 708–712.

74. Crossley, M. L., J. B. Allison & J. B. Muenzen. 1952. II. Treatment of Flexner-Jobling carcinoma in Sprague-Dawley rats with 2,4,6-triethylenimino-s-triazine (TEM). Proc. Soc. Exptl. Biol. Med. **80**: 452–455.

75. Shapiro, D. M., A. Goldin, B. H. Landing, A. D. Bergner, F. Faiman & B. Goldberg. 1949. Cancer chemotherapy: analysis of results of different screening techniques. Cancer. **2**: 100–112.

76. Haddow, A. 1954. Experimental and clinical aspects of the action of various carboxylic acid derivatives in the aromatic nitrogen mustard series. Ciba Foundation Symposium on Leukemia Research. : 196–204. Little, Brown. Boston, Mass.

77. Luck, J. M. 1956. Action of p-di(2-chloroethyl) amino-L-phenylalanine on Harding-Passey melanoma. Science. **123**: 984.

78. Tarnowski, G. & C. C. Stock. 1955. Selection of a transplantable mouse mammary carcinoma for cancer chemotherapy studies. Cancer Research. **15**: 227–232.

79. Carlson, W. W. & C. C. Morgan. 1954. Effect of glycol mesylates on mouse sarcoma 180. Proc. Soc. Exptl. Biol. Med. **85**: 211–213.

80. Shay, H., M. Gruenstein & C. Harris. 1954 The effect of triethylene thiophosphoramide (Thio-TEPA) in the treatment of chronic myelocytic chloroleukemia in the rat. J. Natl. Cancer Inst. **15**: 463–482.

81. Crossley, M. L., J. B. Allison, W. W. Wainio & J. B. Muenzen. 1951. Chemotherapy of tumors in rats. I. The treatment of sarcoma 231 in the King A rat with 2,4,6-triethylenimino-s-triazine and related products. J. Natl. Cancer Inst. **12**: 305–321.

82. Loustalot, P., B. Schär & R. Meier. 1955. Über antimitotische und tumorhemmende Eigenschaften des inneren Salzes des 2,5-Bis-Äthylenimino-hydrochinons. Experientia. **11**: 186.

83. Domagk, G. 1954. Experimental chemotherapy of tumors. Z. Krebsforsch. **59**: 617–622.

84. Sassenrath, E. N., D. C. Morrison, A. M. Larson & D. M. Greenberg. 1955. Evaluation of the carcinostatic activity of 1,2 bis(β-chloroethylthio)ethane. Cancer Research. **15**: 755–759.
85. Yoshida, T., H. Sato, B. Nagasawa, A. Atsumi, H. Sato, K. Kobayashi, K. Hosokawa, K. Nakamura, M. Ishidate, Y. Sakurai. 1950. Experimental studies on chemotherapy of malignant growths using Yoshida sarcoma. I. Gann. **41**: 93–96.
86. Ishidate, M., Y. Sakurai, T. Yoshida, H. Satoh & H. Imamura. 1954. Experimental studies on chemotherapy of malignant growth employing Yoshida sarcoma animals. VI. Effect of N-bis-β-chloroethyl α-amino acids and their derivatives. Gann. **45**: 528–531.
87. Elion, G. B., G. H. Hitchings, C. C. Stock, K. Sugiura & S. M. Buckley. 1952. Tumor inhibition by 2,4-bis(ethylenimino) 6 chloropyrimidine. Cancer Research. **12**: 259.
88. Sugiura, K. Unpublished data.
89. Burchenal, J. H. Unpublished data.

DISCUSSION: PART II

Arthur L. Walpole (*Imperial Chemical Industries Limited, Manchester, England*): We all admire, even if we cannot all emulate, C. C. Stock's extensive and detailed studies of the growth-inhibitory action of alkylating agents upon experimental tumors. Stock has addressed himself to the problem of how to decide which of those substances active against such tumors should be given priority in clinical trial. He has stressed, rightly to my mind, the desirability of testing against a variety of animal tumors those substances that prove of interest in preliminary screening and of comparing their action over a range of dose levels in each case. The results of such investigations, however, represent only one half of an equation, the other half of which must be provided by the clinician. While recognizing the difficulty of achieving comparable objectivity and precision in clinical work, I hope that the clinical studies to be presented in later sessions of the conference will approach in this respect the high standard set for laboratory studies by Stock. This is essential if valid conclusions are to be drawn as to the relationship between the action of inhibitory agents upon experimental tumors on the one hand and upon human malignant diseases on the other.

Hugh J. Creech (*Institute for Cancer Research and Lankenau Hospital Research Institute, Philadelphia, Pa.*): I propose to present a summary* of some recent results of the work† of my associates and myself with various analogues of nitrogen mustard. Several of these compounds have been studied clinically and in the laboratory by other investigators, who report their observations elsewhere in these pages (Jones *et al.* and others).

Our primary study deals with nitrogen mustard derivatives of certain antimalarial-type quinolines; this project was independently conceived by Richard M. Peck and myself, and by Ralph Jones, Jr., during the fall of 1954.

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After some preliminary work at our Institute, Peck, under an American Cancer Society Fellowship, continued the synthetic side of the problem with Alexander Haddow and Franz Bergel at the Chester Beatty Research Institute, London, England, from September 1955 to August 1956. Following our original plan, Peck undertook syntheses designed to produce compounds in which the nitrogen mustard group was directly attached to the quinoline ring; Ralph Jones, Jr., Charles C. Price, and A. K. Sen, at the University of Pennsylvania, Philadelphia, Pa., started to prepare compounds in which the nitrogen mustard group was located at the terminus of the alkylamino side chain of the antimalarials. As a result of our tests with ascites tumors, Peck and Robert K. Preston of the staff of the Institute for Cancer Research in Philadelphia, in close collaboration with Jones and Price, are currently confining their syntheses to the introduction of the nitrogen mustard group into a variety of side chains attached to different quinoline nuclei.

Because of the growth-inhibitory properties of certain quinolines and their specific patterns of tissue distribution, it is our hope that these new nitrogen mustard derivatives may display enhanced antitumor activities and preferential localization in different tissues. Added impetus is given to this study by the recent observations of Alfred Gellhorn and his co-workers that two antimalarials (quinacrine and camoquine) showed activity against brain tumors in tissue culture although they were ineffective clinically, presumably because adequate blood levels were not attainable. From the clinical point of view, it is encouraging that the Chester Beatty Research group found that their phenylalanine and phenylbutyric acid derivatives of nitrogen mustard offered advantages over nitrogen mustard itself because of effectiveness on oral administration and a lessened tendency to provoke side reactions in patients. Nitromin, the *N*-oxide of nitrogen mustard, which has been studied in Japan, is considered an improvement because of low toxicity. We believe that these observations indicate the possibility that the combination of nitrogen mustard with antimalarials may produce potentially important cancer chemotherapeutic agents.

Quantitative comparisons of these compounds for antitumor activity are being conducted by Reed F. Hankwitz, Jr., and myself with the use of mouse ascites tumors. Studies of their effectiveness against leukemia and solid tumors in rats are being made by Jones and Wilhelmina Dunning, pharmacological investigations are being made by Jones and Leon Schmidt, and preliminary clinical trials are being conducted by Jones, Sidney Farber, and others.

Under our experimental conditions, we found that a truly extensive prolongation of the survival time of mice bearing ascites tumors was accomplished by nitrogen mustard, by certain derivatives of nitrogen mustard, and by colchicine. Definite but weak activity was displayed by A-Methopterin and *N*-methylformamide; no more than trace activity, and this only with certain ascites tumors, was shown by 6-mercaptopurine, urethane, 8-azaguanine, and 2,6-diaminopurine. In our studies the two alkylating agents, Myleran and 2-chloroethylmethane sulfonate, have displayed no activity against ascites tumors in mice. The antimalarials, primaquine, quinacrine, and chloroquine, as well as the intermediate glycol of quinacrine nitrogen mustard, also had no effect on the survival time of these mice.

The formulas of some of the compounds we have tested against ascites tumors are given in FIGURE 1. Structure No. 1 is that of phenylalanine nitrogen mustard; No. 2, mannitol nitrogen mustard; No. 3, camoquine mustard; No. 4, chloroquine mustard; and No. 5, quinacrine mustard. Structure No. 6 represents pamoic acid, methylene bis(2-hydroxy-3-naphthoic acid), which forms insoluble salts with the nitrogen mustard analogues. The types of alkylamino side chain being attached to the quinoline nucleus are given in structure No. 7. The nuclei in the compounds currently under study are quinacrine, quinoline-4-, 2-methylquinoline-4-, 2-phenylquinoline-4-, 2-(*p*-chlorophenyl)quinoline-4-, 7-chloroquinoline-4-, and 6-methoxyquinoline-4-. Salts of the nitrogen mustard analogues include hydrochlorides, sulfates, pamoates, naponates, and picrylsulfonates. In our opinion, a careful survey of the structure-activity relationships of a limited number of combinations between these nuclei and side chains should adequately demonstrate any possible enhanced antitumor properties of the antimalarial-type nitrogen mustards.

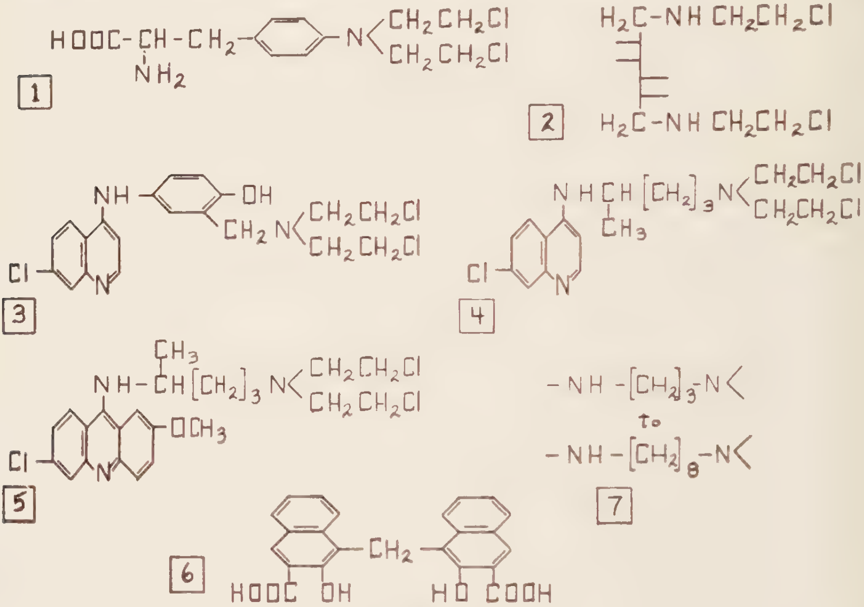

FIGURE 1

Of the 84 compounds now being examined, 6 were supplied by the Chester Beatty Research Institute, 12 by Price and Sen of the University of Pennsylvania, 8 by Parke, Davis & Company, 7 by the Sterling-Winthrop Research Institute, and 38 by Peck and Preston of this Institute. Thirteen miscellaneous compounds are also under study. To date, only one nitrogen mustard derivative of 8-aminoquinoline, prepared by Price and Sen, has been studied. Other analogues of the latter type are being synthesized by Robert C. Elderfield and his group at the University of Michigan.

It is necessary, of course, to adopt and maintain a strict set of satisfactory experimental conditions for any comparative study of antitumor activity. In our standard procedure, the compounds, either in solution or in finely homogenized suspension in saline, were injected intraperitoneally 1, 2, and 3 days after inoculation of 25-gm. Swiss mice* with ascites tumors. Although these analogues are reasonably stable in physiological saline, we have followed the practice of preparing fresh solutions each week. Four different ascites tumors were employed, and large numbers of cells were inoculated to obtain uniform killing times in the control mice and to permit a rigorous trial of the compounds. Information on the experimental conditions and results is presented in TABLE 1. The tumor listed as EF is a hypotetraploid Ehrlich

TABLE 1

SURVIVAL INDICES OF SWISS MICE BEARING ASCITES TUMORS

Compound	Optimum effective range		Therapeutic index	3-Max. survival index			7-Max. survival index
	No. mice	Dosage µM, kg.	(EF-7)	K2 14	ELD 21	EF 7, 18	EF 7
HN2·HCl	476	1–6	4	2.7	2.6	2.6	5.2
L-Ph.al.M	231	25–50	1.5	3.0	2.3	2.7	5.6
D-Ph.al.M	108	50–60	2	2.3	2.0	2.6	4.0
Ph.but.M	112	33–50	1.5	1.6	1.1	2.0	2.8
Naph.M	24	1000	—	—	—	1.5	—
HN2·ox·HCl	41	100–300	2	—	1.0	1.7	—
Mann.M·HCl	60	50–60	2.5	1.9	—	2.0	3.6
6-MeO-4-mebu M·HCl	100	2	1.5	2.7	2.2	1.9	3.7
Cam.M·HCl	209	2–7.5	5	2.2	1.3	1.6	2.7
Quin.M(mebu)·HCl	316	2	4	2.5	1.7	2.4	4.8
Quin.M(propyl)·HCl	188	0.5–1.5	2.5	2.3	1.4	1.9	3.0
2-Me-7-Cl-4-mebu M·HCl	192	1–2.5	2	2.2	2.2	2.0	3.0
7-Cl-4-mebu M·HCl	362	3–7.5	2	2.6	1.5	2.3	4.1
7-Cl-4-hexyl M·HCl	343	2–4	1.5	2.1	1.1	2.0	3.6
7-Cl-4-propyl M·HCl	240	2–5	1.5	1.6	1.8	1.8	3.2
7-Cl-4-ethyl M·HCl	186	8–20	5	1.8	—	1.8	2.7
7-Cl-4-(direct)M·HCl	116	200	—	—	1.0	1.0	—
6-MeO-4-mebu M·pam	154	12–30	7	2.4	1.6	2.5	5.1
6-MeO-8-mebu M·pam	123	50–60	3	2.6	2.1	2.3	4.0
Quin.M(mebu)·pam	48	25–30	2+	2.8	—	2.2	4.0
2-Me-7-Cl-4-mebu M·pam	313	25–40	6+	2.6	2.4	2.8	5.4
7-Cl-4-mebu M·pam	400	15–50	8+	2.6	2.3	2.6	5.0
7-Cl-4-hexyl M·pam	56	25–60	4	2.2	—	1.8	3.4
7-Cl-4-propyl M·pam	124	2.5–16	2.5	2.4	2.9	2.6	5.4
7-Cl-4-ethyl M·pam	70	10–50	6+	2.9	2.1	2.0	3.6

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* I.C.R. Swiss mice raised in our Institute.

clone isolated by T. S. Hauschka several years ago. Control mice inoculated with 7 million cells of this tumor recently have shown a mean survival time of 14 days; with 18 million cells, the mean survival time was 13 days. Since the activities of the compounds against these two inocula of the EF tumor were nearly the same, the results have been combined in the table. Inoculation of 21 million cells of the ELD tumor, a hyperdiploid Ehrlich-Lettré ascites tumor, resulted in a mean killing time of 16 days. An inoculum of 14 million cells of the K2, or Krebs 2, ascites tumor led to a mean survival time of 7 days. The ascites form of sarcoma 37, with a mean killing time of 10 days, was employed also in preliminary studies.

In the heading of TABLE 1, under optimum effective range, are listed only the numbers of mice employed in the tumor-effective dosage levels; about 60 per cent of this total was used in the EF-7 and EF-18 series. An additional 25 to 50 per cent of the listed number of mice was needed to locate this range and to assess the toxicities of the compounds. Since comparisons are being made of analogues of nitrogen mustard, the daily dosages have been expressed in terms of micromoles of compound per kilogram of body weight (μM/kg.). Relatively small variations from the optimum ranges caused a pronounced drop in the survival of the mice. An approximate therapeutic index was determined with mice that had been inoculated with 7 million EF ascites tumor cells. This index expresses the ratio of the daily dosage of compound that, when administered on days 1, 2, and 3, killed 25 to 40 per cent of the mice at 5 days post injection, to the particular dosage within the optimum range that produced the greatest effect on the ascites tumor.

The majority of the experiments were terminated at the expiration of a period that was three times the control mean survival time. Under such conditions, the prolongation of survival time has been expressed in terms of a "3-max. survival index." When all the mice in an experimental group lived to this time of sacrifice, the assigned value became 3.0. A value of 1.0 indicates that the administration of the test compound caused no prolongation of survival time. Since these experiments were deliberately terminated as indicated, the listed values are usually lower than the true ratios of experimental to control mean survival time. Some of the experiments were continued for a period that was seven times the mean survival time of the saline-injected controls; the theoretical maximum for this survival index is 7.0. Mice that survived for this period of time had no signs of ascites tumor at autopsy.

The first seven compounds listed in TABLE 1 have demonstrated some clinical usefulness and serve as special controls. The hydrochloride of nitrogen mustard (HN2·HCl) was effective at low dosage, showed a relatively wide range in the amounts needed for exhibition of high activity against the tumors, and had a good therapeutic index. This compound served as a control for many of the experiments, which accounts for the large number of mice used with the compound. The amounts of the L- and D-phenylalanine mustards (L-Ph.al.M and D-Ph.al.M) and of the phenylbutyric acid mustard (Ph.but.M) required for effective action against the ascites tumors were much greater than that of nitrogen mustard itself, and the therapeutic indices were less favorable. It will be noted that the D form of the phenylalanine nitrogen mustard was less active than the L form; the latter was just as effective as nitrogen mustard in causing prolongation of survival time. The phenylbutyric acid mustard displayed a relatively poor activity against the ascites tumors, and the β-naphthyl nitrogen mustard (Naph.M), even at an extremely high dosage, caused only a slight prolongation of survival time of mice inoculated with the EF-7 ascites tumor. Nitromin (HN2·ox·HCl) also had only a slight effect on the EF tumor at high dosage and did not exhibit the superior therapeutic index claimed by the Japanese investigators. Significant prolongation of survival time was obtained following administration of mannitol nitrogen mustard (Mann.M·HCl; FIGURE 1, No. 2), which has only one β-chloroethyl group per nitrogen.

In general, the most satisfactory prolongations of survival time occurred with mice bearing the EF and K2 tumors. It will be recalled that the control mean survival time of mice with the Krebs 2 tumor was about one half those with the EF and ELD ascites tumors. The ELD ascites tumor responded less satisfactorily, and the results with sarcoma 37 were so consistently poor that they have not been included in the table.

The next group of compounds includes the hydrochlorides of a few of the antimalarial-type nitrogen mustards. It will be noted that the molar ranges of activity of nearly all these analogues were equivalent to that of nitrogen mustard itself. Two of the compounds showed therapeutic indices similar to that of nitrogen mustard; the others had indices equivalent to, or better than, that of the phenylalanine nitrogen mustard. Excellent 7-max. survival indices were displayed by quinacrine mustard [Quin.M(mebu)·HCl], chloroquine mustard (7-Cl-4-mebu M·HCl), and the hexyl homologue of the latter (7-Cl-4-hexyl M·HCl).

The effect of the length of the side chain between the nitrogens was determined in the quinacrine and chloroquine series. In both instances the compounds with the propyl side chain [Quin.M(propyl)·HCl] and (7-Cl-4-propyl M·HCl) were required in lesser amounts for the demonstration of antitumor activity than those containing the methylbutyl side chain [Quin.M(mebu)·HCl and 7-Cl-4-mebu M·HCl]. The propyl derivatives, however, showed relatively greater toxicities than the methylbutyl derivatives and, thus, had less satisfactory therapeutic indices. The methylbutyl analogues displayed better over-all effects than did the propyl derivatives in causing prolongation of survival time. Because of the different pharmacological specificities caused by alterations in the chain length of the antimalarials themselves, it will be of interest to determine whether the propyl and methylbutyl analogues show different patterns of tissue distribution. In the chloroquine series, the analogue with the ethyl side chain (7-Cl-4-ethyl M·HCl) was less toxic than the other members and was equivalent to the propyl analogue in its effect on survival time of the tumor-bearing mice.

It is interesting that the introduction of the nitrogen mustard group directly into the quinoline nucleus at the 4-position led to the production of an inert compound [7-Cl-4-(direct)M·HCl]. The presence of a 2-methyl substituent in the chloroquine mustard structure (2-Me-7-Cl-4-mebu M·HCl) did not cause any substantial alteration in activity. The considerably different side chain of camoquine mustard (Cam.M·HCl; FIGURE 1, No. 3) also permitted retention of antitumor effectiveness. Another analogue (6-MeO-4-mebu M·HCl), in which a 6-methoxy group replaces the 7-chloro group of chloroquine mustard, was effective in prolonging the survival time to a substantial extent. Thus, it is possible to have considerable modifications in the ring and side chain structures of the quinoline carrier of nitrogen mustard and still retain high antitumor activity. As mentioned earlier, it is expected from the results of the antimalarial studies that strikingly different pharmacological properties may be conferred on the nitrogen mustard moiety by these modifications.

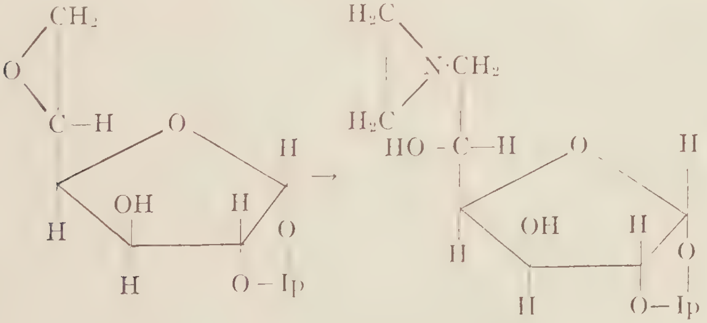
Studies have been conducted also with the extremely insoluble pamoates (FIGURE 1, No. 6) of the antimalarial mustards. As may be seen from TABLE 1, most of these compounds were extremely active against ascites tumors, being equivalent to nitrogen mustard itself in many instances. The pamoates of chloroquine mustard (7-Cl-4-mebu M·pam.), 2-methylchloroquine mustard (2-Me-7-Cl-4-mebu M·pam.), and the 6-methoxyquinoline-4-derivative (6-MeO-4-mebu M·pam.) were particularly effective. In general, the pamoates were much less toxic and had considerably better therapeutic and 7-max. survival indices than the corresponding hydrochlorides. Their overall greater effectiveness against ascites tumors is probably caused by a gradual release of active component from the salt. Considerable difficulty has been encountered in the preparation of pamoates showing the presence of the theoretical amount of chlorine. Extensive research on this problem is in progress. Comparisons among preparations containing different percentages of chlorine have shown that increasingly better activity against ascites tumors is encountered with an increasingly closer approach to the theoretical amount of chlorine. It is anticipated, therefore, that rigorously pure pamoates will display slightly better 3-max. and 7-max. survival indices than those recorded in TABLE 1.

Certain variations have been made from the described standard experimental conditions. In tests with a limited number of compounds, it was found that the activity against the EF-7 ascites tumor in C57Bl mice was approximately 70 per cent of that obtained with Swiss mice bearing this tumor. The compounds were about one half as effective upon intraperitoneal injection at days 3, 4, and 5 after tumor inoculation into Swiss mice as they were at days 1, 2, and 3. Under the experimental conditions employed thus far, the intraperitoneal route of injection has been the only effective procedure with the Ehrlich and Krebs ascites tumors. Although intravenous, intramuscular, subcutaneous, and oral methods of administration resulted in profoundly different degrees of toxicity, there was no prolongation of the survival time of mice bearing these ascites tumors. There are indications from Price's laboratories that the subcutaneous route of administration may be effective against the ascites form of the Gardner lymphosarcoma which is rapidly invasive.

In conclusion, by suitable methods of synthesis, it has been possible to obtain a variety of quinoline derivatives of nitrogen mustard showing high activity against ascites tumors. This leaves us in the fortunate position of having many tumor-active compounds available for selection or rejection according to (1) ease of synthesis and purification, (2) pharmacological specificity, (3) activity against leukemia and solid tumor systems in animals, and (4) preliminary toxicological and therapeutic trial in patients.

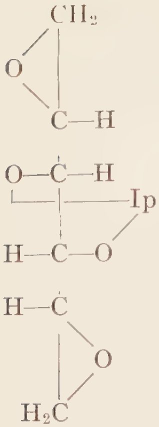
L. Vargha (*Research Institute of the Pharmaceutical Industry, Budapest, Hungary*): Since the discovery of the cytoactive properties of nitrogen mustard by Goodman *et al.*[1] in 1946, numerous synthetic and natural cytoactive substances have become known. All these active substances, with the exception of some amino acid derivatives[2] recently developed, are derivatives of compounds that do not occur naturally in the body. However, it is known that, generally, the cell membrane is easily permeated by substances that take part in cell metabolism; for example, the sugars and the natural amino acids. We have therefore assumed the task of synthesizing suitable derivatives of substances of this kind in the hope of finding among them cytostatic agents that will act more effectively or more selectively than those already known. Our efforts were directed first to the sugar group; we endeavored to synthesize ethylenimino and β-dichlorodiethylamino derivatives of sugars and of sugarlike substances, with special reference to the fact that other ethylenimine and nitrogen mustard derivatives are among the most potent cytoactive substances. Ethylenimino derivatives of sugars seemed to be of interest also from a chemical point of view, as the three-membered heterocyclic ring is capable of manifold conversions that have not yet been studied in the sugar group.

The epoxides of sugars and sugar alcohols appeared to be suitable starting materials for the synthesis of such ethylenimino derivatives, as the epoxides form amino compounds with ammonia and organic bases as a result of opening of the anhydro ring. It was supposed, therefore, that ethylenimine, being slightly basic, would react in a similar manner. At first we chose as sugar moiety the 1,2-isopropylidene-5,6-anhydro-D-glucofuranose (I) and the 1,2,5,6-dianhydro-3,4-isopropylidene-D-mannitol (III) described originally by Ohle and myself[3] and by Wiggins,[4] respectively.

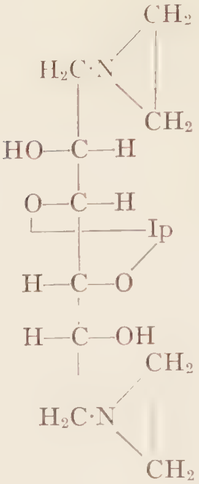


I

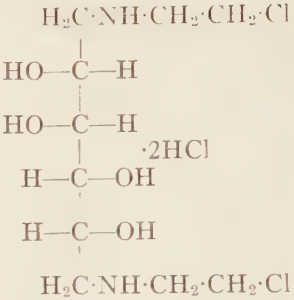
II



III



IV



$$\begin{array}{c} H_2C\cdot NH\cdot CH_2\cdot CH_2\cdot Cl \\ | \\ HO-C-H \\ | \\ HO-C-H \\ | \\ H-C-OH \\ | \\ H-C-OH \\ | \\ H_2C\cdot NH\cdot CH_2\cdot CH_2\cdot Cl \end{array} \cdot 2HCl$$

V

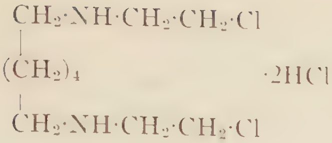
The experiments have fulfilled our expectations. The reaction of I and III with ethylenimine took place at room temperature, was exothermic, and yielded compounds II and IV, respectively.

The glucose derivative (II) is a very stable crystalline compound, but its attached isopropylidene group cannot be removed without simultaneously opening the ethylenimino ring. Consequently, we were unable to synthesize the free 6-ethylenimino-glucose.

The mannitol derivative (IV), which is obtained in the form of a tough syrup, is very unstable as contrasted with the glucose derivative (II) because, within a few days, it polymerizes to a glassy mass quite insoluble in water. Freshly prepared, however, on addition of hydrochloric acid the substance gives the well-crystallized-dihydrochloride (V) with simultaneous breaking of the acetal bond and opening of the ethylenimino ring.

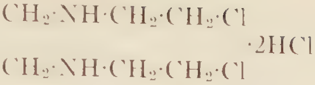
This mannitol derivative (V) disclosed interesting biological properties (to be dealt with in detail later), and it was therefore subjected to some alterations for the purpose of studying the relationships between its structure and its biological activity. For example, because we were interested in the significance of the alcoholic hydroxyl groups, we prepared hydroxyl-free analogues of V; that is, the hexane derivative (VI) and the ethane derivative having four carbon atoms less (VII). It was also interesting to examine the influence of the configuration of the carbon chain upon the cytoactivity. To this end we synthesized the dulcitol derivative (VIII), a stereoisomer of the mannitol derivative (V).

To investigate further the connection between structure and biological activity, we prepared the amides formed with β-chloroethylamine of gluconic acid (IX), of glucosaccharic acid (X), and of mannosaccharic acid (XI). Both diamides (X and XI) show a certain similarity to V; each of these compounds bears at both ends of the molecule the same β-chloroethylamino group, although with different bonds.



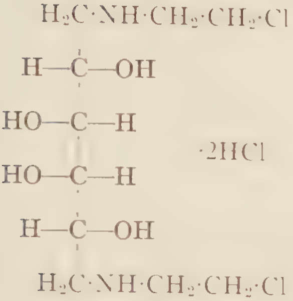
$$\begin{array}{l} CH_2{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}Cl \\ | \\ (CH_2)_4 \qquad\qquad \cdot 2HCl \\ | \\ CH_2{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}Cl \end{array}$$

VI

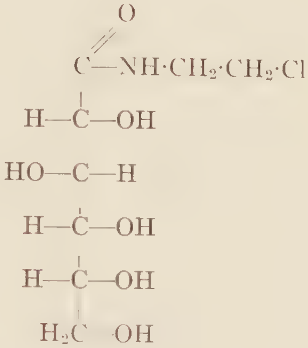


$$\begin{array}{l} CH_2{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}Cl \\ \qquad\qquad\qquad\qquad \cdot 2HCl \\ CH_2{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}Cl \end{array}$$

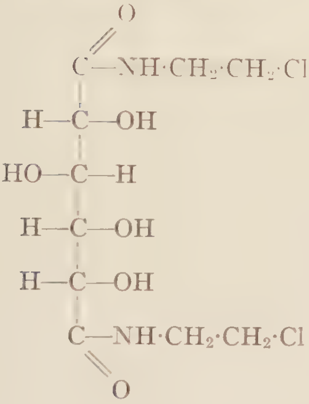
VII



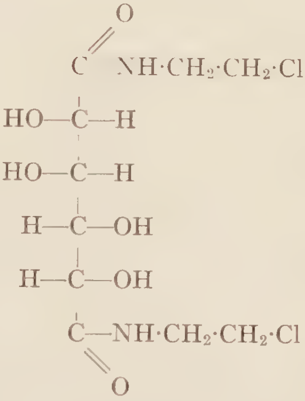
VIII



IX



X

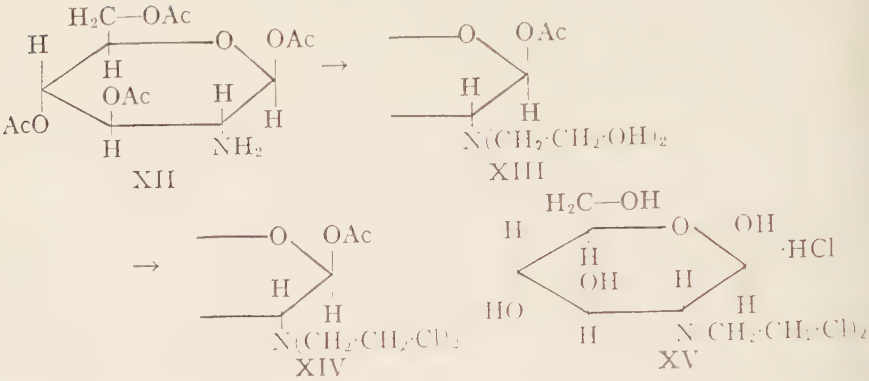


XI

The compounds mentioned above may not be considered real nitrogen mustard derivatives because they lack a β-dichlorodiethylamino group with the tertiary nitrogen atom characteristic of the nitrogen mustard compounds.

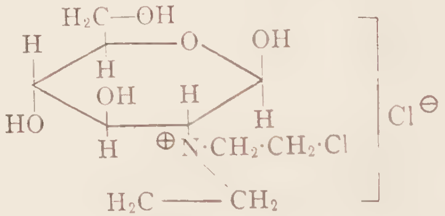
The natural D-glucosamine seemed to be a suitable starting material for the synthesis of nitrogen mustard analogues of the sugar group. A publication of Quastel,[8] later reported to be erroneous,[9] should be mentioned. Quastel stated that D-glucosamine itself should be able to inhibit tumor growth. In order to obtain a nitrogen mustard analogue derived from D-glucosamine, we transformed tetra-*O*-acetyl-D-glucosamine[10] (XII) through intermediates XIII and XIV into the desired *N*-bis(β-chloroethyl)-D-glucosamine (XV).

This nitrogen mustard-derivative of D-glucose (XV) is slightly stable in aqueous solution. Half of its chlorine content becomes converted into



XII XIII XIV XV

chloride ions within a few days. It may be supposed that the imonium cation (XVI) arises here, as has been proved by Hanby and his collaborators[11] in the more simple tertiary β-chloroethylamino derivatives. In contrast to the glucosamine derivative (XV), the mannitol and dulcitol derivatives (V and VIII), which contain a secondary nitrogen atom, remain for months in aqueous solution without decomposing.



XVI

All the compounds mentioned, except the diethylenimino derivative (IV) which, while otherwise interesting, was unstable, have been screened in the Hungarian Oncological Institute, Budapest, for their cytoactive, that is, tumor growth-inhibiting capacity, and for their possible clinical use. The results of these experiments and also the favorable clinical responses to BCM, 1,6-di-(2-chloroethylamino)-1,6-deoxy-D-mannitol dihydrochloride (V, BCM, Degranol), are reported in the summaries of Kellner *et al.*,[12] Sellei *et al.*,[13] and Kellner and Németh.[14]

The correlation between structure and biological activity may be summarized briefly as follows: BCM (V) constitutes a new type of compound among the known tumor-inhibiting agents that act by biological alkylation; in contrast to nitrogen mustard and its analogues, it is a secondary base. This difference manifests itself in some of the compound's biological properties. The presence of hydroxyl groups seems to be essential to this type of compound, as BCM's hydroxyl-free analogue (the hexane derivative, VI) lacks any cytostatic effect. Furthermore, the carbon configuration likewise plays a role, as proved by the fact that the dulcitol derivative (VIII), stereoisomeric with the mannitol derivative (V), was completely inactive. Finally, it may be established that substitution of the methyl group in nitrogen mustard by a glucose residue, as in compound XV has little influence on the biological (cytostatic) properties of the same compound.

References

1. Goodman, L. S., M. M. Wintrobe, W. Dameshek, M. J. Goodman, A. Gilman & M. T. McLennan. 1946. J. Am. Med. Assoc. **132**: 126.
2. Bergel, F. & J. A. Stock. 1954. J. Chem. Soc. : 2409.
3. Ohle, H. & L. Vargha. 1929. Ber. deut. chem. Ges. **62**: 2435.
4. Wiggins, L. F. 1946. J. Chem. Soc. : 384.
5. Haworth, W. N., W. G. M. Jones, M. Stacey & L. F. Wiggins. 1944. J. Chem. Soc. : 61.
6. Gensler, W. J. & G. R. Thomas. 1951. J. Am. Chem. Soc. **73**: 4601.
7. Haskins, W. T., R. M. Hann & C. S. Hudson. 1942. J. Am. Chem. Soc. **64**: 137.
8. Quastel, J. H. & A. Cantero. 1953. Nature. **171**: 252.
9. Lettré, H. 1953. Naturwissenschaften. **40**: 513.
10. Bergmann, M. & L. Zervas. 1931. Ber. deut. chem. Ges. **64**: 975.
11. Hanby, W. E., G. S. Hartley, E. O. Powell & H. N. Rydon. 1947. J. Chem. Soc. : 519.
12. Kellner, B., L. Németh & C. Sellei. 1955. Naturwissenschaften. **42**: 582.
13. Sellei, C., S. Eckhardt, F. Hartai & B. Dumbovich. 1956. Lancet. **1**: 785.
14. Kellner, B. & L. Németh. 1956. Z. Krebsforsch. **61**: 165.

L. Németh, B. Kellner, and K. Lapis (*National Oncological Institute, Budapest, Hungary*): Combinations of sugars with different ethylenimino and chloroethylamino compounds have been prepared by Vargha[1] and by B. Dumbovich. Among these, 1,6-di-(2-chloroethylamino)-1,6-deoxy-D-mannitol dihydrochloride (Degranol, BCM) possessed the most favorable properties and, consequently, detailed tests were made biologically and on different animal tumors.[2] The examinations included comparisons with other known biological alkylating agents. In this paper we have recorded the data obtained in these investigations.

Material and Methods

Wistar, Chester Beatty, and Debrecen rats; Phylaxia, C3H, DBA, AKR mice; rabbits; dogs; and guinea pigs were used. Transplantation was subcutaneous, intraperitoneal, and intravenous. Body weight was recorded daily and the tumors were measured with calipers. Blood was drawn from the tails of the rats, from the ears of the rabbits, and from the jugular vein. Specimens of bone marrow were taken by femur and tibia punctures. Formalin was used for fixation and hematoxylin-eosin for staining. The blood-smears and bone marrow were stained with Giemsa; stains of ascites tumor cells were made by the Giemsa, Feulgen, and Papanicolaou methods.

Pharmacology and Toxicology

Degranol is readily soluble in water, giving a weakly acid aqueous solution that remains stable even after standing at room temperature for a prolonged period. Dilute solutions of 1 per cent or less have no local irritating effect on the skin or on the mucous membranes (conjunctiva). Degranol does not irritate the gastric mucosa, and is equally effective when administered orally or parenterally, intravenously or intraperitoneally (TABLE 1).

As shown in TABLE 2, of the agents tested, Degranol possessed the highest therapeutic index, the lowest cumulative residual toxic effect and, when given in therapeutic doses, proved the most beneficial.

TABLE 1
TOXICITY OF DEGRANOL AND HN2
(MG./KG. I.V.)

	Animal	LD_{100}	LD_{50}*	Maximum tolerated dose (MTD)	Therapeutic dose	Minimum effective dose (MED)	MTD/MED
Degranol...	Rat	160	80	50	10	1	50
	Mouse	200	90	50		1	50
	Rabbit	100	50	25	10	1	25
	Dog	100	50	20	5	1	20
	Guinea pig	20					
HN2.......	Rat	3.0	1.6	1.0	0.8	0.1	10

* Determination of LD_{50} according to Behrens; 10 animals per dose; observation, 21 days.

TABLE 2
TOXICITY OF DIFFERENT MUSTARD DERIVATIVES

	Degranol	HN2 oxide	HN2	HN3	TEM	Sarcolysin
LD_{100}, mg./kg...........	160	130.0	3.0	2.2	1.1	50
LD_{50}....................	80	80.0	1.6	1.25	0.9	25
MTD....................	50	40	1.0	0.8	0.25	20
MED....................	1.0	1.0	0.1	0.2	0.1–0.2	
Chemotherapeutic index, MTD/MED...........	50	40	10	4	2.5	
Average therapeutic dose..	5–10	5–20	0.8	0.2–0.3		5
Residual cumulative effect after 24 hours, per cent[3]	25	45	75	90	40	

Sensitive tumors (Yoshida, Walker) were cured generally by small doses (simultaneous administrations of 5 to 10 mg. in 4 to 5 cases) of Degranol; established tumors required higher doses and repeated injections and, even then, in many cases, only prolongation of the life span was achieved. Guerin tumors also could be cured occasionally. Crocker tumors and mouse ascites tumors were inhibited only by toxic dose levels. In instances of lymphoblast leukemia (AKR-Paterson), permanent remission, normalization of blood picture, and disappearance of enlarged lymph nodes and of hepatosplenomegaly could be attained with low doses.

TABLE 3

TUMOR-INHIBITING EFFECT OF DEGRANOL ON DIFFERENT TUMORS

Guerin carcinoma 10 × 10 mg. every 24 hours	Ninety per cent inhibition Occasional cure	With tumor sizes greater than 5 gm., effect uncertain I.V. metastasis arrested
M-1 sarcoma 10 × 15 mg. every 24 hours	Fifty per cent inhibition	Metastasis arrested
Walker carcinoma 5 × 10 mg. every 24 hours	Cure Ninety to 100 per cent inhibition	
S-45 sarcoma 5 × 16 mg. every 72 hours	Cure	Evaluation difficult because of spontaneous regression of tumor
Yoshida ascites 5 × 10 mg. every 24 hours	Cure Span of life prolonged—100 per cent	
S-Benevolenskaja 10 × 10 mg. every 24 hours	Twenty-five per cent regression	
Brown-Pearce carcinoma 10 × 10 mg. every 24 hours	Tumor size unmeasurable	Metastasis arrested
Ehrlich carcinoma solid 10 × 10 mg. every 24 hours	Less than 25 per cent regression	
Ehrlich ascites 5 × 10 mg. every 24 hours	Twenty to 50 per cent regression	Prolongation of span of life only attainable in animals inoculated with suspension pretreated with Degranol
Crocker sarcoma 10 × 15 mg. every 24 hours	Fifty per cent regression	Attainable only by high doses of 15 to 20 mg.
Lymphosarcoma, mouse 10 × 10 mg. every 24 hours	Twenty-five to 50 per cent regression	
Spontaneous mammary carcinoma 10 × 10 mg. every 24 hours	Without effect	
Hepatoma 10 × 10 mg. every 24 hours	Without effect	
Mouse leukemia (AKR-Paterson) 5 × 5 mg.	Permanent regression attainable even with low doses (5 mg.)	
Benzpyrene mouse-skin carcinoma 10 × 10 mg. every 24 hours	Without effect	

TABLE 4
COMPARATIVE DATA ON THE INHIBITING ACTIVITY OF DEGRANOL AND OTHER ANTICARCINOGENIC AGENTS

Tumor	HN2	Degranol	Mitomen	Sarcolysin	Imino-chinon	TEM	Myleran	Mercapto-purine	Podo-phyllin	Colcemid
Yoshida	+++	++++	++++			++				
Walker	+++	++++	++++	++++	++++	+++	+	+		
Guerin	+	+++	++	++	++	++	±	±	0	0
Crocker	±	++	+++	+		±	0	+++		
M-1	±	++		+						
S-45	++++	++++	++++							
C3H spontaneous mammary	0	0	0							
Ehrlich ascites	±	++	++	+		++	0	+		
Ehrlich solid	+	+								
Brown Pearce		+		+	+		+	±		+
Leukemia AKR		++++								

Much depended on the manner of administration. Best results were attained with simultaneous administration, either immediately after transplantation or after 24 hours. Injections at intervals of 24 hours were most beneficial (for leukemia, 5 mg./kg.; for solid tumors, 10 mg./kg.). When Degranol was administered every 48 hours the body weight and blood picture were unaffected. A total quantity of 100 mg. introduced within 10 days had toxic effects. From the eleventh to the twenty-first day onward, a certain resistance to the agent developed. This resistance was of less consequence here than it was with other anticancer agents, such as triethylene melamine (TEM) and folic acid antagonists. When it was transplanted, the resistant tumor could be influenced even in successive passages by usual doses of Degranol.

Weekly injections of high doses (50 mg.) exerted a similar tumor-inhibiting activity, and the bone marrow could regenerate during this period. Nevertheless, experiments with such high doses were unsuccessful because, although the life span was prolonged, the animals died from different organic causes (liver, kidney, or adrenocortical insufficiency).

TABLE 3 demonstrates the efficiency of Degranol over a wide spectrum of tumors. In particular, it arrested the "take" and spread of tumor cells, but it also exerted an inhibitory effect on the growth of established tumors. Its favorable toxicological properties accounted for the infrequency of harmful side effects.

As evidenced by the data presented in TABLE 4, different kinds of transplanted tumors responded differently to the drugs. There were sensitive tumors, such as the Walker 256, the Yoshida ascites tumor, and the S-45 rat sarcoma. Induced leukemias were also very sensitive. Guerin and Ehrlich ascites tumors were of medium sensitivity. M-1 rat sarcoma and Brown-Pearce rabbit carcinomas were fairly resistant, while the spontaneous mammary carcinomas and induced tumors showed the strongest resistance.

Summary of the Effect on the Blood Picture

On administration of Degranol, the first changes occurred in the blood and blood-forming organs. In normal animals, leukopenia developed in cases treated with high doses (25 to 50 mg.) 24 hours after administration; and after 3 to 4 days on smaller dosage (10 mg.). TABLES 5 through 9 show the results in mice, rats, and rabbits of various treatments with Degranol.

The first effect was a drop in the number of lymphocytes; the eosinophils disappeared at an early stage; later the granulocytes also disappeared. The initial leukopenia was followed sometimes by mild leukocytosis and, when high doses (100 mg.) were given, leukocytosis of several hours' duration occurred. The young forms, mainly the young lymphocytes, showed the greatest sensitivity. In the bone marrow also the lymphocytes and eosinophils were the first to disappear, then immature forms of leukocytes in the order of their aging. The plasma reticulum and the endothelial cells of the sinuses were the most resistant. Basophilic cells were still present in the bone marrow when the latter was almost completely depleted. Red blood

TABLE 5

EFFECT OF A SINGLE DOSE OF DEGRANOL ON THE BLOOD PICTURE OF NORMAL MICE

Dose (mg./kg.)	WBC before injection / after 4 days (thousand)	Gran.:Ly.:Eos.	Bone marrow
10	4.0 3.6	18:80:2 50:40:2	-
25	7.0 3.3	25:72:1 70:22:1	Disappearance of eosinophils
50	6.8 2.1	15:80:3 90: 5 :0	Depletion of 25 per cent
100	6.2 2.8	20:78:2 80:12:1	Depletion of 50 per cent
150	7.0 2.5	30:68:1 98: 0 :0	Depletion of 75 per cent
200	4.8 0.4	30:56:1 96: 0 :0	Depletion of nearly 100 per cent
250	6.0 0.0	40:52:2 0: 0 :0	Total depletion

TABLE 6

EFFECT OF A SINGLE DOSE OF DEGRANOL ON THE BLOOD PICTURE OF RATS (100 MG./KG.)

Hours	0	1	2	3	6	10	24
RBC (million)	8.2	7.8	7.2	7.4	7.7	8.0	7.0
WBC (thousand)	23.0	16.0	19.0	11.0	10.0	7.0	5.2
Gran. (per cent)	7	22	35	39		68	80
Eos. (per cent)	3	1	1	0		0	0
Ly. (per cent)	88	67	65	61		32	18

Mild leukocytosis after initial leukopenia, followed by death after 36 hours because of leukopenia, eosinopenia, and lymphopenia.

cells and thrombocytes were destroyed in the bone marrow and disappeared in the peripheral blood, but not until later.

Compared to other chloroethylamino derivatives, the hematological action of Degranol was more protracted than that of HN2. Degranol appeared to exert the strongest action on lymphocytes; its effect was invariably stronger than that of HN2 oxide or of sarcolysin.

TABLE 7

EFFECT OF PROTRACTED TREATMENT WITH DEGRANOL ON THE BLOOD PICTURE OF RATS, 6 × 50 MG. EVERY 7 DAYS*

Days	0	2	4	7	14	21	28	35	42	49
WBC (thousand)	21	9	9.6	13	17	12	18	20	15	17
Gran. (per cent)	10	82	80	64	73	58	38	27	33	30
Ly. (per cent)	89	16	18	26	25	32	51	60	65	70
Eos. (per cent)	1	0	2	3	2	1	0	3	1	0

* Such dosage spared the blood picture and the bone marrow, but lymphopenia remained.

TABLE 8

EFFECT OF PROTRACTED TREATMENT WITH DEGRANOL ON THE BLOOD PICTURE OF RABBITS, 7 × 10 MG./KG.

	RBC	WBC	Gran.	Ly.	Eos.
Control	6.0	4.6	30	64	2
After 7 days	4.2	1.2	100 Twenty per cent distorted toxic granules	0	0
After 20 days	3.7	0.6			

Death after 21 days.

TABLE 9

EFFECT OF ORALLY ADMINISTERED DEGRANOL ON THE BLOOD PICTURE AND BONE MARROW OF RABBITS

	Dose	RBC before, and after treatment (million)	WBC before, and after treatment (thousand)	Gran.:Ly.:Eos.	Death	Bone marrow
Rabbit	10 × 5 mg./kg. daily by intubation	4.8 4.0	6.8 3.2	30:68:2 90:10:2	Survived	Twenty-five per cent depletion
Rabbit	10 × 25 mg.	4.8 3.9	7.0 1.2	25:70:2 88:10:0	Death after 10 days	Ninety per cent depletion

The proper dosage of Degranol, as of all biological alkylating agents, is not yet established. Insofar as tumor-inhibiting activity is concerned, doses of 5 to 10 mg. doses administered every 24 hours and, eventually, every 48 hours, seemed to be the most beneficial. As for protecting the bone marrow, periods of 72 to 96 hours were favorable. In the case of certain tumors good results could be attained even with high weekly doses (50 mg.) without affecting the bone marrow.

The Effect of Degranol on Mouse Leukemia

The effects of Degranol on mouse leukemia (AKR-Paterson) were examined in two ways: (1) its effect on animals with established signs of leukemia enlarged lymph nodes, hepatosplenomegaly, and leukocytosis; (2) its effect in prolonging survival in young animals injected intraperitoneally with suspensions of leukemic spleen.

Animals of the first group became free of symptoms after having received 5 doses of 5 mg. each. The number of white blood cells dropped from 40,000 to 80,000 to 4000 to 5000, the spleen and liver decreased in size, and the lymph nodes disappeared completely, as did infiltrations of other organs and of the intestinal wall.

Lymphocytosis of nearly 100 per cent dropped to between 5 and 1 per cent, young forms disappeared completely, and the white blood cells contained mainly granulocytes. There was also a moderate drop in red blood cells (from between 6 and 7 million to 4 million). This state was attained after 5 to 10 I.V. injections, and it lasted more than 3 months. Afterward, the number of leukocytes rose again in one group of animals and, after some months, distinct leukemic symptoms again developed. Repeated treatment proved effective once more. In some cases the treatment was less effective. When receiving doses of 10 mg. kg., the animals often died with symptoms of agranulocytosis and bone marrow depletion. The developed leukemia of adult animals was not always connected with a significant rise of leukocytes. Distinct lymph node enlargement was often accompanied by leukocyte values of from 20,000 to 25,000; it occurred also in normal mice and continued over a long period. Degranol was harmful in treating leukemic mice with lymph-node enlargement and stable blood picture, and these animals died within a short time after the initiation of such treatment. In many cases of animals with few clinical symptoms, often it could not be determined at autopsy whether they were leukemic at all. Nevertheless, for all untreated adult animals, autopsy showed a general glandular hyperplasia always absent in treated animals. Leukemia of the adult animals had much in common with human chronic lymphoid leukemia, and the successful animal experiments made it seem most likely that Degranol exerts a similar effect on the pathological overgrowth of the human lymphatic system. Later this supposition was proved true in the case of human patients.

In every case in the second group, the development of acute leukemia could be arrested by simultaneous administration of 5 doses of 5 mg. kg. every 24 hours.

Histological Changes Caused by Degranol in Organs and in Tumors[4]

Dissection of animals killed by treatment with Degranol revealed passive hyperemia of the organs. The spleen became smaller and the lymph nodes disappeared almost entirely. Marked inflation of the duodenum and small intestines was caused by accumulation of gases and liquids. The mucous membrane became hyperemic, and local punctate hemorrhages could be observed.

The most significant cytological changes always could be noted in tissues of high mitotic index and, therefore, wherever cell turn-over was rapid (in the mucous membrane of the duodenum, in the lymphatic system, in the blood-forming organs, and in the testes). At the outset of the action, mitoses were damaged, and the number of all the dividing forms diminished to 1 to 2 per cent. During the strongest action, many specific distorted mitoses could be observed, amounting sometimes to 10 to 15 per cent of the total cells. The number of amitoses and polynuclear tumor cells increased even more rapidly, amounting to as much as 50 to 75 per cent. In vascular areas of the tumor, extensive dissolution of pyknotic cells and nuclei took place. Giant cells were still predominant in the recovery phase, which seemed to lead to a new cell population. The initial period lasted 6 to 12 hours. The damage caused during maximal activity passed on to the recovery phase in 48 to 96 hours, the end of which could not be stated accurately. After 5 to 10 days, traces of the changes were still distinguishable.

The morphologic picture was greatly influenced by the dosage. The histological picture was radically different if the same amount of drug was administered in single doses or repeatedly. The frequency of repeated doses was also of importance.

The Effect of Degranol on the Metastasis of Transplanted Tumors[5]

The metastasis-inhibiting activity of Degranol manifested itself both with subcutaneous and intravenous injection (TABLE 10).

Carcinogenic Effect of Degranol

In mice treated with very low doses, sometimes after the thirtieth week but usually only after the fiftieth week, pulmonary adenoma could be produced in 3 animals out of 25, fibrosarcoma in 2, and lymphoid leukemia in 2. Experiments with rats failed to give the same results.

Summary

Degranol (BCM), synthesized by Vargha and Dumbovich, proved to be superior to nitrogen mustard with regard to both hematological and tumor-inhibiting effect. It was less toxic and had a wider therapeutic range than nitrogen mustard. The histological changes and courses produced by the two agents in organs and tumors were essentially similar. The effect of Degranol was more protracted than that of HN2. Degranol exerted its activity primarily on blood and lymphoid elements, and favorable therapeutic results could be attained most rapidly in instances of experimental

Table 10

Metastasis—Inhibiting Effect of Degranol (Guerin Carcinomas)

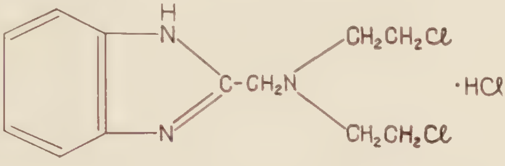
	Animals with metastases	Distribution of metastases							
		Superficial lymph nodes	Mediastinal lymph nodes	Abdominal lymph nodes	Lung	Kidney	Liver	Adrenal glands	Ovary etc.
Control 18 animals 5 million/cu.mm. tumor cells I.V.	18	18	18	18	6	3	0	2	2
Degranol 10 animals 6 × 20 mg./kg. I.V.	0	0	0	0	0	0	0	0	0
Nitromin 10 animals 6 × 20 mg./kg.	3	3	3	1	0	0	0	0	0
Cortisone 9 animals 10 × 2 mg./rat S.C.[1]	9	2	9	7	9	3	7	7	3
Cortisone + Degranol 10 animals	0	0	0	0	0	0	0	0	0

mouse leukemia. When administered over a prolonged period to animals with transplanted tumors, it caused serious morphologic alterations followed by the destruction of a great part of the sensitive tumors, and it arrested the growth of the resistant tumors, with consequent prolongation of the life span of the animals. In animal experiments, it exerted a significant inhibiting action on metastases developing in regional areas of subcutaneously transplanted tumors, as well as on metastases following the I.V. injection of carcinoma cells. Compared with other alkylating agents (HN2, TEM, Myleran, and Nitromin), Degranol appears to have the most favorable inhibiting effect on metastases.

References

1. Vargha, L., L. Toldy, Ö. Fehér & S. Lendvai. 1957. Synthesis of new sugar derivatives of potential antitumor activity. J. Chem. Soc.: 805–809.
2. Kellner, B. & L. Németh. 1956. 1,6 Bis(β chloraethylamino)-1,6 desoxy-D mannit dichlorhydrat (BCM), ein neues Stickstofflostderivat. Z. Krebsforsch. **61**: 165–179.
3. Druckrey, H. 1956. Vergleichende Prufung der chemotherapeutischen Wirkung von N-oxyd-Lost und anderen alkylierenden Substanzen auf Tumoren von Ratten. Arzneimittel-Forsch. **9**: 539–550.
4. Kellner, B. 1957. Histological changes induced in transplanted tumors by nitrogen mustard, mustard gas and BCM. Acta Morphol. Acad. Sci. Hung. **7**(4).
5. Lapis, K. & L. Németh. 1956. Die Wirkung des BCM auf die Metastasenbildung transplantierter Tiergeschwülste. Klin. Wochschr. **34**(31-32): 864–867.

Erich Hirschberg, Alfred Gellhorn, and William S. Gump (*Institute of Cancer Research, Columbia University College of Physicians and Surgeons,* *New York, N. Y., and The Givaudan Corporation, Delawanna, N. J.*): In this monograph numerous alkylating agents are presented that have the same bifunctional reactive group but differ in the carrier to which this group is attached. We desire to introduce a new nitrogen mustard in which the carrier is benzimidazole, a putative purine antagonist.



2-[Di-(2-chloroethyl)aminomethyl] benzimidazole
hydrochloride

FIGURE 1. Structure of benzimidazole mustard.

The structure of the compound is given in FIGURE 1. A 5-day toxicity test in mice established the daily maximum tolerated dose at 8 to 9 mg./kg. The daily dose chosen for the evaluation of carcinostatic activity[1] against a variety of solid tumors was 5.0 to 6.5 mg./kg. by the intraperitoneal route. At these dosage levels, benzimidazole mustard caused statistically significant inhibition of tumor growth in 4 of 4 experiments with mammary adenocarcinoma 755, in 4 of 6 experiments with sarcoma 180, in 4 of 7 experiments with mammary adenocarcinoma E 0771, and in 1 of 3 experiments with glioma 26. In tests with the Ehrlich ascites carcinoma,[2] in which the agent was administered intraperitoneally at 8.0 mg./kg. for the first 3 days after tumor transplantation, the survival time of the mice was prolonged significantly in 5 of 6 instances. The ascitic form of mouse leukemia L 1210 responded to daily intraperitoneal injections at 5.0 mg./kg., but subcutaneous administration at 5.0 or 10.0 mg./kg. was ineffective.

TABLE 1
COMPARATIVE EFFECTS OF NITROGEN MUSTARDS ON VARIOUS MOUSE TUMORS

Mustard	Ehrlich	S 180	755	E 0771
Benzimidazole	+	+	+	+
Quinacrine	+	−	−	−
Camoquin	+	−	−	−
Chloroquine	−*	−	−	−
CB 1348	−*	−	−	−
HN2	+†	+‡	−	±§

* These two compounds, while negative under our conditions, have been reported by Creech to be inhibitory to this tumor.[3]

† Data in our series are incomplete. The results are quoted from Creech *et al.*[2]

‡ Not tested here. The results are quoted from Clarke.[4]

§ Not tested here. The results are quoted from Sugiura.[5]

This widespread activity of benzimidazole mustard in a mouse tumor spectrum contrasted with results obtained concurrently with a number of other nitrogen mustard derivatives. In TABLE 1, in very abbreviated form, our comparative results with these agents are summarized. The only tumor in this group that responded to acridine and quinoline mustards was the Ehrlich ascites tumor; data on a large series of derivatives with this tumor have just been reported.[3] Almost without exception, the three solid tumors failed to respond to the nitrogen mustards included in these experiments.

The use of benzimidazole as the carrier of the nitrogen mustard moiety, then, appeared to endow this agent with more pronounced carcinostatic activity and, perhaps, with a more favorable therapeutic index in these animal experiments. The details of this work and the results of preclinical pharmacology are to be reported elsewhere.[6]

References

1. GELLHORN, A., A. KELLS & M. GOLINO. 1955. Investigation of diverse systems for cancer chemotherapy screening. VI. Mammary adenocarcinoma 755, glioma 26, and Brown-Pearce carcinoma. Cancer Research. Suppl. No. **3**: 38–43.
2. CREECH, H. J., T. S. HAUSCHKA, R. F. HANKWITZ, JR., B. J. LITTLETON & J. ANDRE. 1955. Investigation of diverse systems for cancer chemotherapy screening. VIII. Studies with four distinct lines of the Ehrlich ascites tumor for the evaluation of twenty-seven potential inhibitors. Cancer Research. Suppl. No. **3**: 47–51.
3. CREECH, H. J. 1957. Discussion: Part II. Biological effects of alkylating agents. Ann. N. Y. Acad. Sci. **68**(3): 868.
4. CLARKE, D. A. 1955. Investigation of diverse systems for cancer chemotherapy screening. II. Mouse sarcoma 180. Cancer Research. Suppl. No. **3**: 14–17.
5. SUGIURA, K. 1955. Investigation of diverse systems for cancer chemotherapy screening. III. Studies in a spectrum of mouse and rat tumors. Cancer Research. Suppl. No. **3**: 18–27.
6. HIRSCHBERG, E., A. GELLHORN & W. S. GUMP. 1957. Laboratory evaluation of a new nitrogen mustard derivative, 2-[di-(2-chloroethyl)aminomethyl]benzimidazole. Proc. Am. Assoc. Cancer Research. **2**: 213. *See also* Cancer Research. **17**: 904.

Part III. Clinical Results in Cancer

NECESSARY CRITERIA FOR THE COMPARATIVE EVALUATION OF ALKYLATING AGENTS IN CLINICAL CANCER CHEMOTHERAPY

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In Denmark, a reputable medical journal published a report on the clinical efficacy of external radiation combined with the ingestion of fresh egg yolk in the treatment of osteogenic sarcoma; in Great Britain, injections of an extract of urine have been said to cure inoperable gastric carcinomas; in Cuba, cytochrome *c* is thought to be capable of restoring terminal cancer patients to their families and businesses; in the United States, we are told through privately published articles that synthetic lipids cause a diminution or even disappearance of an increasing proportion of almost all types of advanced malignancies, or that the extract of blood serum of horses previously injected with a preparation from a mold culture is biologically active in the management of a significant number of patients with cancer. If these allegations were even in part correct, it is possible that our attention today would not be focused on the alkylating agents, but would be devoted to considerations of classes of compounds presently not recognized as valuable in cancer therapy. Why have we ignored these dramatic claims? The explanation can be found in the fact that the proponents of the treatments failed to apply acceptable objective criteria in their evaluations, so that the conclusions reached are unreliable. Such results confuse, distort, and depreciate the field of cancer chemotherapy. I should like to review briefly an organization of criteria that may be helpful in the comparative evaluation of alkylating agents used in the treatment of human neoplastic diseases.

The estimation of benefit from a therapeutic regimen rests on a tripod: the clinical material, the drug, and the response. Each leg must be carefully described if the results obtained are to have meaning within a series of cases treated at one institution or if the evidence reported is to be interpretable and comparable by other institutions.

Clinical Material

The first leg of the tripod is the characterization of the clinical material before experimental therapy is initiated. Convincing evidence for improvement is dependent upon an accurate base line. Three aspects should be defined in this description of the clinical material: *(1)* the tumor characteristics, *(2)* the tumor distribution, and *(3)* the effect of the tumor on the host.

TABLE 1 shows these three facets with some of the parameters of value under each. It is obviously not feasible in a general discussion such as this to be explicit for each neoplastic disease. A comprehensive knowledge of the biology and natural history of the tumor is a prerequisite to guide the pretreatment work-up. Nevertheless, some of the points itemized here may be emphasized.

Tumor characteristics. Under this heading morphology is placed first. This is to emphasize the necessity for a specific histological diagnosis, a necessity frequently overlooked by the exponents of treatments mentioned at the outset of this paper and, unfortunately, overlooked on occasion even by clinics represented in these pages. Morphology is significant not only for specific tumor diagnosis, but also for its indication, in many instances, of the type of natural history or the therapeutic response that may be anticipated. Thus, for example, it is important to characterize the lymphosarcomas precisely, because the giant follicular form of the disease behaves, in general, far more benignly than the reticulum cell (lymphoblastic, large cell) type. Confusion and contradiction can be avoided by an accurate pathological appraisal. It is entirely possible that some of the discrepancies in the literature on the nitrogen mustard therapy of bronchogenic carcinoma rest on the fact that the frequency of objective improvement is greater in the anaplastic tumors than in the well-differentiated squamous cell types. If an author does not specify the distribution of morphologically separable tumors within each tumor category, then it may be impossible to interpret the significance of his result.

Table 1
Clinical Material

(A) *Tumor characteristics*
  (1) Morphology
  (2) Growth rate
  (3) Past response to therapy
  (4) Effect on host economy

(B) *Tumor distribution*
  (1) Route of extension
  (2) Sites of involvement

(C) *Host status*
  (1) General
    (a) Physical
    (b) Psychological
    (c) Work performance
  (2) System function
  (3) Past and current therapy

Summary classification of clinical material
Tumor biology
Anatomical dispersion
Tumor-host relationship

A definite conception of the tumor growth rate is another essential baseline parameter. The investigator must address himself to this issue explicitly in order to avoid fooling himself and others. How many times have we not seen patients with disseminated neoplastic disease growing very slowly and apparently existing in symbiosis with the patient? This is to be contrasted with the tumor that metastasizes explosively and widely, so that each new site can be observed to increase in size and extent over periods of days. Although the anatomical distribution of the tumors and even their morphologic features may be comparable, their biological characteristics are obviously different.

The previous response to treatment is an important characterization. It will indicate biological behavior favorable or unfavorable to treatment; it will suggest whether the tumor is still responsive or becoming refractory; it will forewarn of susceptibility to noxious side reactions from the drug to be used.

An ever-broadening body of evidence is being formed on the pathophysiology of the host associated with neoplastic disease and probably due to the tumor. Increased rate of red cell destruction, modifications in carbohydrate and mineral metabolism, and depression of protein synthesis are among the more recently described alterations. The characterization of the tumor in terms of relative villainy, that is, greater or lesser malignancy, may be made more precise by defining its effects on host economy. This form of mensuration, as well as the others mentioned, provides categorization within a disease entity, and thereby facilitates more accurate comparisons of the clinical material on which therapeutic trials have been conducted.

Tumor distribution. The distribution of tumor, both in terms of its route of spread and its anatomical localization, is of self-evident importance in the pretreatment evaluation.

Effect of the tumor on the host. The status of the host is also of obvious significance in evaluating a therapeutic regimen. This is particularly important in the use of alkylating agents in a patient with apparently localized disease, as in the use of Nitromin with surgery for operable gastric cancer, and also in a patient who is preterminal with widely disseminated tumor. The general physical state and the work performance of the patient provide a static and dynamic description of the host. The psychological characterization of the patient before treatment is worthy of note for sake of completeness, but it is my conviction that changes in the state of the psyche following therapy should receive no consideration in the evaluation of the regimen, except under those circumstances in which the drug may produce a toxic psychosis. At a recent meeting in New York, N. Y., on the value of hormone modification in the therapy of cancer, a distinguished visitor from abroad said, in effect, that the preoccupation with objective changes in the tumor overlooked the prime concern of doctors, which was to make patients feel better. He was proud of the fact that the over-all effect of the procedure he employed was to improve the patient's spirits, appetite, and sense of well-being, and he was impatient with those who emphasized tiresomely that the frequency of objective tumor regression was distressingly low. We are as pleased as he when patients feel better, but the objective of therapeutic research in cancer is to control the tumor and, as a consequence thereof, to improve the psyche otherwise we might all better spend our time looking for a more effective hashish.

An appraisal of organ and system function provides a base line for the evaluation of drug toxicity and, when tumor involvement has brought about disturbances, the return, or failure to return, to normal following treatment constitutes an impressive objective criterion of therapeutic effects. The system functions to be measured and recorded before therapy will be influenced in part, of course, by the disease under investigation.

A major source of error in the interpretation of chemotherapeutic trials is the failure to note or recognize the importance of immediately preceding or concomitant treatment measures. By now we should be sensitized to the fact that there is a lag in the full demonstration of radiotherapeutic effect in many diseases and that, accordingly, we must be careful not to give credit to a chemotherapeutic regimen that is initiated closely following X-ray treatments. Too many cases of foul, fungating breast cancers have been thought to be dramatically benefited by one form of presumed definitive treatment or another when, in fact, supportive measures such as local and systemic antibiotics, blood transfusions, and encouragement given concomitantly are the real but unrecognized factors in the improvement. It is well to question critically whether the drug under study is solely responsible for the effects observed or whether additional and uncontrolled measures have been introduced simultaneously.

Having carried out the pretreatment evaluation of the clinical material, it should be possible for the investigator to characterize and group it, not only with regard to disease entities, but also, within the latter, according to the biology of the tumor, its anatomical distribution, and the status of the tumor-host relationship. With this information available, we can proceed to the second leg of the chemotherapy evaluation tripod, namely, the drug.

The Drug

The items presented in TABLE 2 suggest the type of information we should like to have about the drugs to be discussed. From the chemical considerations already presented in this monograph, we recognize the interrelationships of the various alkylating agents. If there are quantitative and or qualitative differences among these drugs, structure-activity correlations should be evident. If further exploration within the alkylating group is to be continued, it is probable that the material presented within these pages will indicate the type of chemical synthesis that would be most profitable.

TABLE 2
THE DRUG

(A) Chemistry
(B) Experimental tumor chemotherapy
(C) Route of administration and dosage
(D) Tissue or cell distribution
(E) Metabolism and excretion
(F) Toxicity
(G) Mechanism of action

Pharmacological summary
Deviations from nitrogen mustard

Since one alkylating agent may affect a certain human tumor, while another does not, we shall be expected to examine the clinical evidence critically and also to call on all ancillary information that may contribute to this important issue. High among the latter is the evidence for antitumor activity on transplantable tumors in experimental animals. The laboratory chemotherapy studies can be controlled better than the human therapeutic trials and, therefore, it should be possible to demonstrate qualitative antitumor differences among the alkylating agents, if any such exist. If among several alkylating agents there is similarity in antitumor effects on a spectrum of experimental neoplasms and apparent differences on a variety of human cancers, we should be cautious in accepting the conclusion that there is a qualitative difference in these agents in the treatment of human disease. The more likely explanation is that the clinical observations have been less well made than the animal experiments.

The alkylating drugs now available are administered by the oral, intravenous, intra-arterial, intramuscular, intracavitary, and intratumor routes. The multiple ways in which the drugs may be given, depending in part on their chemical configuration, constitute one of the main distinguishing features among the compounds. This frequently determines the preference for one over another. In my estimation the intracavitary route of administration does not constitute real cancer chemotherapy. Even though very useful on occasion, it is a technique for the palliation of a local problem and has no significance in modifying the natural history of the tumor under treatment. I, for one, find it impossible to justify the intratumor injection of a drug. This, it seems to me, is a giant step backward. More about this appears elsewhere in these pages and, perhaps, new evidence will be presented to alter such a categorical indictment.

Although there is considerable variation in dosage employed for the members of the alkylating group, this is really of little importance since, at most, the quantities are small and, with therapeutic doses of all of the drugs, they are uncomfortably close to the toxic dose.

In considering the wisdom of further research on the alkylating agents, evidence for significant differences in tissue or cell distribution would provide a powerful stimulus. As we proceed in our investigations it will be important to look for suggestions that this is the case. Such differences are strongly indicated by the variation in the emetic action of many of the mustards. This is most reasonably explained by the failure of agents such as chlorambucil or triethylene thiophosphoramide to attain sufficient concentration in the vomiting center.

Metabolism and excretion do not appear to be important factors in the biological action of the alkylating compounds now available. The material presented by Creech and Heidelberger may modify this conclusion.

We must pay close attention to what we are told about the comparative toxicity of the alkylating drugs. With the exception of the recognized variation in local irritant activity and stimulation of the medullary emetic center, it may be suggested that there are also quantitative and even qualitative differences with regard to toxicity on bone marrow function. The evidence for the latter in man must be carefully scrutinized. Does Myleran selectively depress granulocytopoiesis in contradistinction to triethylene melamine, for example? In chronic myeloid leukemia both of these agents effectively suppress the granulocyte precursors; when Myleran is used in the treatment of other diseases where dosage is necessarily larger, it has a pancytopenic effect. Thio-TEPA has been said by some to be relatively less toxic than nitrogen mustard. This is the case only if the drug is given in small doses widely spaced. Even then cumulative toxicity is consistently evident when the therapeutic course is compressed into 5 to 10 days. Another type of toxicity less well known than the usual bone marrow depression is observed following regional intra-arterial or intravenous administration. There has been a revival of interest in this type of administration of the alkylating agents in connection with the chemosurgery program (about which we read such interesting accounts in the newspapers), and it will be important for us to learn, from the investigations reported here, of the immediate and delayed side reactions that may follow.

In the preceding pages we have read about the mechanism of action of the alkylating compounds. In the following pages it will be of interest to note whether the clinical evidence suggests that these drugs differ significantly in their mechanism of cytotoxicity.

The Response

Now that we have considered the pretreatment evaluation of the clinical material and have indicated the characterizations of the drug under study, we are ready to discuss the third leg of the critical appraisal tripod in cancer chemotherapy: the response. In TABLE 3 are listed five major headings under which the response may be critically summarized. Let us assume for the moment that we are agreed that cancer chemotherapy with the alkylating agents should be reserved for patients with disseminated disease. Let us assume further that the objective of cancer chemotherapy is the destruction and/or inhibition of the total tumor. Under these conditions the primary requirement for a positive effect is that there be evidence of an antitumor effect at the major sites of involvement without evidence of progression at any other site. This definition requires, then, that each patient be evaluated individually, and objective improvement means total improvement in the disease under study. It may seem tiresome, indeed, to have this definition restated; however, one cannot but be impressed by the continued summarizing in the literature of inhibitory effects on specific types of tumor involvement in a series of patients to the point that it becomes impossible to determine what actually happened to each individual in the series. For example, in such compilations it is possible for 40 per cent of lung metastases to decrease, 60 per cent of skin nodules to disappear, and 20 per cent of liver metastases to shrink while, at the same time, in the 50 patients studied, not

TABLE 3
THE RESPONSE
Summary of Objective Evidence of Antitumor Effect and Hazards of Therapy

- (A) *Modification of tumor progression*
  - (1) Anatomical evidence
  - (2) Biochemical evidence
  - (3) Functional evidence
- (B) *Time parameters*
  - (1) Onset of effects
  - (2) Duration of remission
  - (3) Influence on survival
- (C) *Reproducibility*
- (D) *Toxicity*
- (E) *Reasons for failure*

one may have been significantly benefited since, while a skin nodule was decreasing, a lung metastasis was growing and a liver deposit was making its first appearance!

Having introduced the over-all characteristics of a favorable response to treatment, we may categorize the objective criteria under modification of tumor progression. These are self-evident, and brief mention need be made only of the functional evidence. This may be interpreted to mean not only changes in specific distorted functions such as neurological deficits, but also the restoration of the patient to or toward his normal activities. This refers to the performance status as described by Karnofsky.

The time parameters are useful, and they distinguish the effects produced by members of the alkylating group of compounds. For example, the onset of effect is usually fastest with intravenous nitrogen mustard and slower with many of the orally administered congeners. It is well known how difficult it is to define the duration of remission, and yet some effort to quantify this period is helpful in assessing the significance of the therapeutic regimen. Thus, for example, many clinics have observed that nitrogen mustard produces an objective antitumor effect in a significant proportion of patients with bronchogenic carcinoma but, in the majority of instances, the duration of the remission is so transient as to fail to justify its recommendation as a routine.

The influence on survival is of great importance ultimately. At the present stage of effectiveness in cancer chemotherapy, however, this is not a very important criterion of objective improvement. Thus, for example, it has been shown that the addition of nitrogen mustard to the therapeutic aramentarium for Hodgkin's disease has not improved the survival over that observed before its introduction. In spite of this, the usefulness of the drug in the management of this disease is generally acknowledged. There is one other situation in which survival statistics constitute the chief measurement of therapeutic effectiveness of alkylating agents. This applies to the use of these drugs in the chemosurgery program. I believe there is little justification in employing a chemotherapeutic regimen early in a neoplastic disease unless there is convincing evidence that the treatment has an antitumor effect in an advanced stage of its natural history. Nevertheless, this procedure is being carried out in Japan, and I understand that interest in it is finding expression in the United States.

Reproducibility. Anyone who has treated cancer patients has experienced a great feeling of triumph when a patient has responded unexpectedly well to a drug regimen. Unfortunately, after we may have reported this single triumph we try the same drug in the same disease in other patients and nothing happens. From this we must learn that an important part of our chemotherapy evaluation depends upon demonstrating reproducibility. It appears that, in several instances in this monograph, attention to reproducibility of results has been neglected, so that conclusions on the value of chlorambucil in multiple myeloma, of thio-TEPA in breast cancer and cancer of the gastrointestinal tract, and of other compounds rest on unconvincing evidence. We must not forget that naturopath Harry M. Hoxsey produced a remarkable regression of a single neuroblastoma with cascara sagrada and potassium iodide.

Now let us observe how the toxicities of the alkylating agents compare. It is well to remember that the apparent hematopoietic toxicity of these agents is influenced by previous treatment. Before we accept the fact that one drug is less toxic than another, let us be sure that we are making valid comparisons. Let us also weigh the toxic effects with the therapeutic benefits. I shall always remember the remarkable publication of a case of lymphosarcoma cured by nitrogen mustard. The cure was well documented by necropsy, death having been caused by complete bone marrow destruction.

In the comparative evaluation of the therapeutic efficacy of the alkylating agents it may be helpful to consider the reasons for failure as well as for success.

This completes an introduction to criteria that are valuable in the critical evaluation of cancer chemotherapy. I hope they may be helpful in assessing the evidence presented in these pages. From this information we can hope to learn the present place of alkylating agents in the treatment of human neoplastic disease and the future potentialities of these drugs in clinical therapy.

SUMMARY OF RESULTS OBTAINED WITH NITROGEN MUSTARD IN THE TREATMENT OF NEOPLASTIC DISEASE*

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Methyl-bis(β-chloroethyl)amine hydrochloride is known by several names: as the prototype *nitrogen mustard*, by the military code name HN2, and by the Merck trade name Mustargen. It has been used since 1943 in the treatment of patients with neoplastic disease. Methyl-tris(β-chloroethyl)amine hydrochloride (HN3) was the first nitrogen mustard used clinically. In 1942 Gilman *et al.*[1] prepared a report, necessarily under conditions of military secrecy, on the effects produced by the intravenous administration of HN3 in six patients with neoplastic disease. The doses used caused a considerable degree of host toxicity, and a striking regression of enlarged nodes was observed in a patient with lymphosarcoma. In 1943 L. O. Jacobson initiated clinical trials with HN2, and he found marked improvement in patients with Hodgkin's disease. Between 1943 and 1946 several clinics in the United States—in New Haven, Chicago, Boston, Salt Lake City, Portland, Oreg., and New York—cooperated, still under a mantle of secrecy, in a clinical assessment of HN2. By the time the military restrictions were removed in 1946 a total of 160 patients had been treated, and the results were summarized by Rhoads,[2] Chairman of the Committee on Growth of the National Research Council, Washington, D. C., for publication in June 1946. Subsequently, detailed reports appeared from Goodman,[3, 4] from Jacobson[5, 6] and their associates, and from the group at Memorial Hospital in New York.[7] In Rhoads' report,[2] the following general statements were made concerning the therapeutic value of nitrogen mustards:

"(1) They are not a cure for such neoplastic diseases as have been studied.

"(2) In sufficiently large doses they are injurious to many types of tissue; they appear to exert their greatest effects on rapidly growing tissue, presumably either normal or neoplastic.

"(3) Their predominant toxicological effect is damage to normal hematopoietic function. The extent of this injury is the limiting factor in determining the amount that can be given to an individual. In some cases the hematopoietic injury exceeds the effect on the tumor.

"(4) The tumor regressions induced by these compounds (even with maximum dosages) are temporary, with maximal persistence rarely extending beyond several months.

"(5) In many respects their effects are similar to those of X rays. It should be noted, however, that the great advantage of radiation therapy is that it can be given locally."

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The original reports contained an assessment of the therapeutic efficacy of HN2 that has not been altered appreciably by subsequent experiences. In 1942 in Manchester, England, Wilkinson and Fletcher began independent clinical trials with HN2 and HN3; their results in 18 patients were reported in 1947.[8]

Because of the toxic effects of the nitrogen mustards and their limited therapeutic activity, a controlled clinical study was organized in 1946 to determine their indications in various types of neoplastic disease, and to create independent groups of clinical investigators experienced in the use of agents of this type.[9] HN2 was selected as the most promising substance. The drug was supplied by the Chemical Warfare Service, Washington, D. C., and, under the direction of the Committee on Growth, Merck & Co., Inc., Rahway, N. J., packaged and distributed HN2 for clinical use. More than a hundred institutions participated in this cooperative study, and many reports were published during the years 1947 to 1949. As other forms of alkylating agents became available for clinical study, the volume of published work on HN2 diminished. It was interesting to learn from Augustus Gibson, of Merck & Co., Inc., however, that 120,000 10-mg. vials of Mustargen, equivalent to 30,000 courses of treatment, were sold in 1956; this is to be compared with 20,000 vials (5000 treatment courses) distributed during the years 1946 to 1947, the heyday of the clinical evaluation studies on HN2. This rise in the general use of HN2, which has occurred despite the widespread acceptance of other alkylating agents, such as triethylene melamine (TEM), chlorambucil, triethylene thiophosphoramide (Thio-TEPA), and 1,4-dimethane sulfonoxybutane (busulfan), indicates the important role that these agents play in the present-day management of neoplastic disease.

The purpose of this review is to present the dosage, routes of administration, toxicity, and intrinsic therapeutic activity of HN2 in various forms of neoplastic disease, a subject extensively reviewed in the past.[10-20] These observations can be compared later to those presented on the other alkylating agents.

Properties of HN2. HN2 hydrochloride is a white, crystalline powder, water-soluble, and stable in an acid medium. Contrary to general belief, HN2 hydrochloride loses activity slowly in aqueous solution. Under alkaline conditions, however, it iminizes rapidly and reacts with water and many other inorganic and organic substances.

Method of administration and dosage. HN2 is usually made up in a fresh solution containing 1 mg. cc. in physiological saline. It is usually given intravenously into the tubing of a running saline infusion; it can be injected rapidly. HN2 has been injected into the pleural space, the peritoneal cavity, the pericardial cavity, into tumors, into the carotid, hepatic, and femoral arteries, into the aorta, and into the portal vein. To my knowledge, it has not been given orally. During World War II HN3 was given by mouth to 7 human volunteers. Single doses of 4 to 6 mg. day produced a moderate degree of nausea, vomiting, headache, and diarrhea that persisted for 24 hours. This reaction cleared as soon as the drug was stopped.[21] One mg. 3 times daily to a total dose of 15 to 18 mg. in 6 or 7 normal individuals produced a definite degree of leukopenia. This would presumably be in the range of a therapeutic dose and would suggest efficient absorption, since the intravenous leukopenic dose of HN3 in the adult is about 0.3 mg./kg. HN3 caused no definite injury to the intestinal tract, and vomiting was presumably due to the central action of the absorbed drug. The doses used were relatively large. It is conceivable that HN2 may produce therapeutic effects when given by mouth at small and tolerated doses.

A number of toxic effects and complications may follow the intravenous injection of HN2. Thrombophlebitis may occur in the injected vein; this is particularly common when venous pressure is elevated. A severe local reaction results from the extravasation of HN2. A regular and early effect of HN2 is nausea and vomiting which, in our experience, occurs in about 80 per cent of the patients. It seems to occur at all doses in the therapeutic range, although the larger a single dose, the earlier the nausea and vomiting begins. After a single dose of 0.4 mg. kg. intravenously, vomiting may commence in 20 minutes. This reaction usually subsides within a few hours, and may be alleviated in part by barbiturates and chlorpromazine. Less common reactions are a metallic taste immediately following injection, anorexia, weakness, drowsiness, headache, diarrhea, and fever.

The major toxic effect of HN2 is depression of normal hematopoietic function, which is appreciated most readily by examination of the peripheral blood. Within 7 to 10 days, and sometimes sooner, there is a fall in the leukocyte count, followed by a decline in platelets. Maximum depression of these elements usually occurs within 2 to 3 weeks, and is followed by prompt recovery. Excessive doses cause thrombocytopenia, severe bleeding manifestations, anemia, and fever; these events may be fatal. In patients with normal bone marrow function, 15 or more courses of HN2 have been given at appropriate intervals, usually 6 or more weeks apart, without cumulative injury to the bone marrow. If there is prior bone marrow injury or a primary hematological disturbance, ordinary doses may cause profound and prolonged marrow depression. This may be due, in part, to the impaired regenerative capacity of the diseased bone marrow. There are no antidotes to bone marrow injury produced by HN2, but blood transfusions and antibiotics may sustain the patient until recovery begins. Adrenal steroids may diminish the bleeding tendency.

The intra-arterial injection of nitrogen mustard produces evidences of local injury such as erythema, depilation, and edema.[22] HN2 has been injected into the hepatic artery in man without producing appreciable liver injury.

The single-dose LD_{50} of HN2 by the intravenous route in man is estimated to be about 1.0 mg. kg. The usual therapeutic dose, initially suggested by Jacobson, is 0.1 mg. kg. intravenously daily for 4 days. This dose causes some degree of leukocyte and platelet depression within 2 to 3 weeks, and the majority of patients recover promptly. Where impaired bone marrow activity due to previous treatment or to disease suggests unusual sensitivity, the dosage is usually in the order of 0.1 to 0.2 mg./kg.; further treatment is given in 1 to 2 weeks on the basis of the clinical response and the peripheral-blood and bone marrow picture. Some patients with chronic lymphatic leukemia and lymphosarcoma are unusually sensitive to HN2, and severe and prolonged leukopenia and thrombocytopenia may follow the usual course of treatment. In patients with bulky lymphosarcoma or chronic leukemia, a rapid course of HN2 may produce hyperuricemia due to rapid lysis of tissue, with resultant renal impairment. Small spaced doses are therefore advisable. In Austria, Klima and Hornisher[23] have used small repeated doses of HN2, in the range of 0.6 mg./day for a total dose of 40 mg. in patients with lymphomas and leukemia. Nausea and vomiting were not noted, and their dose schedule approximates that recommended for busulfan and chlorambucil.

Larger doses have been tolerated by patients with carcinoma; 0.8 mg./kg. has been given to some patients, particularly with lung cancer, in a single course of treatment, but it is difficult to predict the individuals who will tolerate these large doses.

The dose of HN2 injected into the body cavities or intra-arterially is usually in the range of 0.4 mg./kg., largely for reasons of safety since, even if all the drug reached the systemic circulation, the amount is not hazardous. Generally, however, larger doses can be tolerated by these routes without depression of the peripheral blood count.

Therapeutic Effects of HN2

Quantitative measures of the therapeutic effects of HN2 in various forms of cancer are difficult to delineate precisely. At best one can present only a picture, with a certain outline, emphasis, and coloration, rather than a series of numbers that can be used for quantitative comparisons. The reasons for this are discussed by Gellhorn elsewhere in this monograph, but since HN2 is regarded by many as the base-line alkylating agent, I should like to indicate the complexity of the problem in my own words.

The most important factor is the patient. Cancer runs a unique course in each patient. There is adequate evidence to show that HN2 does not appreciably modify the basic course of a neoplasm, but it may temporarily suppress some of the manifestations of the disease. Brilliant results, therefore, may be obtained in patients treated early in their disease, or in whom it is progressing slowly; conversely, in patients with advanced disease resistant to adequate conventional therapy, or where the process is highly malignant, the response is usually poor. Patients must be classified as to disease type, pattern of dissemination, rate of progression, adequacy of previous treatment, and response to previous therapy. The use of these criteria must be reiterated constantly, despite their obvious relevance in an evaluation study.

To avoid the complex situation presented by the patient, measurements of discrete lesions have been used in order to obtain data suitable for comparative purposes. Some lesions fluctuate spontaneously in size. Other lesions, presumably because of a high rate of cell turnover—that is, cell death versus new cell formation—may show prompt regression only to recur with equal rapidity. The latter lesion cannot be properly compared with another type, perhaps more sensitive, that regresses slowly, but for a longer period of time.

It is important also to emphasize *consistency of response* in specific situations. The use of a dramatic isolated response to illustrate the effectiveness of an agent, may give one drug, because of a fortuitous event or by virtue of a unique biochemical property of the tumor or of host metabolism, an unjustified reputation for specific activity. The total experience on an adequate series of patients must be reported.

Therapeutic responses should be recorded, not only in terms of a tumor regression or of hematological or biochemical changes, but also as it affects the patient's total life situation.

In regard to the drug, a proper trial requires the administration of adequate treatment; in resistant patients, in the case of nitrogen mustard, this means the production of a moderate to severe temporary depression of normal hematopoietic activity. Further factors in the evaluation to be considered are the convenience of administration of the drug, its acceptability to the patient, and safe dosage control. We can try to present a series of pictures of the therapeutic activity of HN2 in specific forms of neoplastic disease. It is inevitable in clinical medicine to find exceptions to every generalization. Also, in the absence of general standards or criteria of response, the word "response" means different things to different observers. The objectivity permitted by the leukemias is one of the major exceptions.

Leukemia[24]

Acute leukemia. In acute leukemia, HN2 does not induce hematological remissions, such as may occur with the folic acid antagonists, with 6-mercaptopurine, or with the adrenal cortical steroids. The leukocyte count can be reduced and, in some cases, relief of bone pain and symptomatic improvement have occurred.

Chronic myelocytic leukemia. HN2 can regularly produce a satisfactory hematological response in early cases of chronic myelocytic leukemia. Improvement consists of a fall in the leukocyte count, a decrease in the size of the enlarged spleen and liver, and a spontaneous rise in hemoglobin. A single course produces hematological improvement for 1 to 4 months, with an average duration of 2 months; when the leukocyte count begins to rise, a second course may be given. In some cases maintenance doses of 0.2 to 0.3 mg. kg. of HN2, often fractionated, have been given at 2- to 9-week intervals to maintain an even clinical situation. These patients often require and tolerate large amounts of HN2. As the disease enters the resistant or late stage, the results with HN2 become less satisfactory. There is little evidence that optimal HN2 therapy substantially prolongs life. Individual remissions from X-ray therapy to the spleen are of longer duration than those produced by single courses of HN2. Occasionally a patient, unresponsive to splenic radiation, responds to a systemic agent such as HN2. Generally, HN2 has not proved to be the most convenient alkylating agent for the control of chronic myelocytic leukemia.

Chronic lymphocytic leukemia. The response of chronic lymphocytic leukemia to HN2 is rather unpredictable; considerably more so than the response of chronic myelocytic leukemia. The mild process in relatively

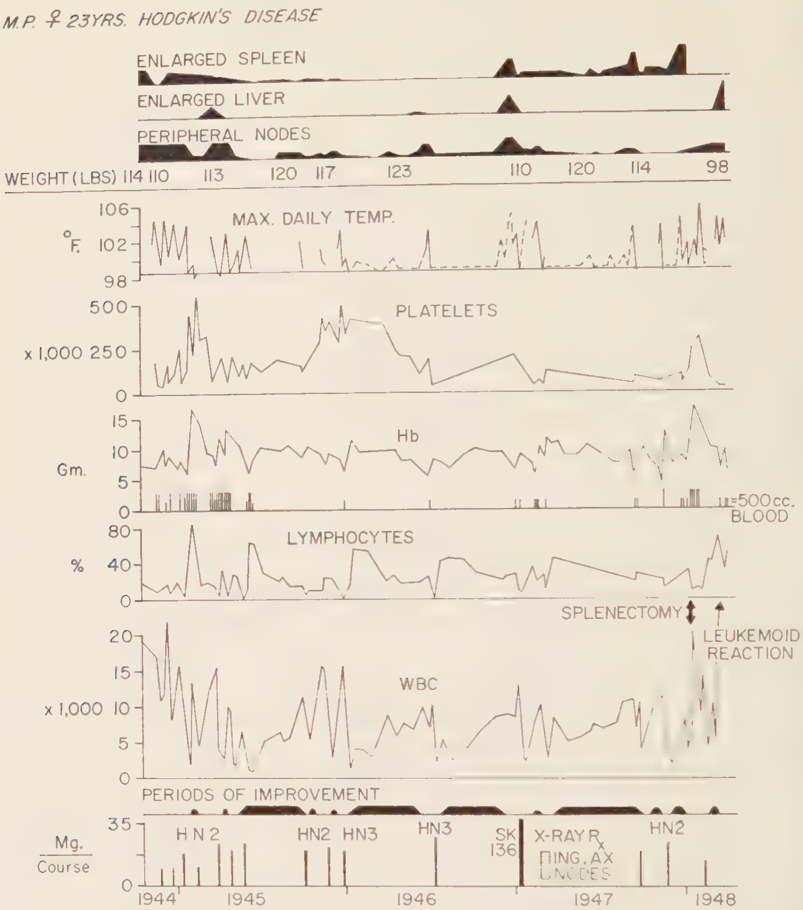

FIGURE 1. The clinical course of M. P., a 23-year-old female, with Hodgkin's granuloma. The disease appeared clinically in March 1944 with chills, fever, and enlarged cervical and axillary nodes. X-ray therapy at this time resulted in regression of the enlarged nodes and clinical improvement for 5 months. In October 1944 the disease recurred, with fever, anemia, generalized lymphadenopathy, and splenomegaly. Small amounts of HN2 were given intermittently from November 23 to February 14, 1945, with only brief periods of improvement. In March 1945 the patient received an HN2 dosage of 0.47 mg./kg. and, associated with the marked leukopenia, there was a 6-month clinical remission, with decrease in lymphadenopathy, splenomegaly, and fever; the need for blood transfusions ceased. She relapsed in September 1945, and a second series of HN2 injections was given. Again the periods of clinical improvement were brief, but finally, following a course of HN3 (0.37 mg./kg.), which produced a severe leukopenia, a 6-month remission occurred. This response was considered to be due to the cumulative effect of the polyfunctional alkylating agents, rather than to the greater therapeutic activity of HN3. This interpretation is supported by the initial experiences with HN2 in this patient, where a prolonged response occurred after a dose of HN2 sufficient to produce a severe leukopenia was given. A second course of HN2 caused a second remission of approximately 6 months. There was a brief response to the trial of another alkylating agent, SK 136 [1,3 diamino-tetrakis(N,N,N',N'-2-chloroethyl)propane]. Subsequently, the patient received local

asymptomatic patients generally shows a satisfactory hematological response, and it may continue for many months after a single course of treatment. Patients in the late stage of the disease, with anemia, thrombocytopenia, and bleeding manifestations are improved only rarely, although favorable responses have been recorded. The patient with chronic lymphocytic leukemia may be extremely sensitive to HN2; conventional doses sometimes have produced a marked leukopenia with severe and sometimes fatal depression of the remaining normal hematopoietic elements. HN2 should be started, therefore, at small doses, and increased after tolerance to the drug has become evident. In other situations neoplastic lymphocytes may be unusually resistant to HN2 and, in attempting to reduce the leukocyte count, severe depression of the normal hematopoietic elements has resulted. Occasionally, the reverse has been true, and a prolonged hematological remission has been produced with a spontaneous rise in hemoglobin level and platelet count. The unpredictable response and the hazards of excessive dosage have caused HN2 largely to be discarded for the treatment of chronic lymphocytic leukemia in favor of the slower administration of the oral alkylating agents.

Lymphomas

Hodgkin's disease. The most useful application of HN2 has been in the treatment of Hodgkin's disease. This drug has exerted a marked suppressive action at many stages of the disease. Its characteristic therapeutic effects are seen as a decrease in size of enlarged lymph nodes, liver and spleen; relief of itching, fever, anorexia, and weakness; and improvement in the anemia often associated with the disease. The prompt detoxification of an acutely ill patient is a remarkable action of HN2, suggesting that it interrupts the production of a substance noxious to the host. The initial course of HN2 will produce appropriate evidence of improvement in 80 to 90 per cent of patients; the remissions may last for from 2 weeks to a year or more, but the average period of improvement is 6 to 8 weeks following a course of treatment. Occasionally several courses of treatment have been necessary before a maximal response is obtained; thus the drug is pushed in resistant patients as far as the blood picture will permit. This is illustrated in FIGURE 1, a clinical record of one of the early patients treated with HN2.[7]

The duration of improvement varies with the inherent activity of the disease; in our experience, the length and degree of improvement following an adequate course of HN2 is of prognostic value. Complete failure or a brief therapeutic response to an adequate course of treatment is an ominous sign, while a prolonged period of improvement is favorable. The

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X-ray therapy to enlarged inguinal and axillary nodes, with a good systemic response. In the last few months of her life she developed severe thrombocytopenia, and a splenectomy was performed in the hope of improving the hematological situation so that further HN2 could be given. This was only of transient benefit, and the patient died, 4 years after the clinical onset of her disease.

intrinsic activity of HN2 in Hodgkin's disease and its indications in relation to other forms of treatment may be summarized briefly:

(1) In general, HN2 does not substantially prolong life,[25] although undoubtedly it has been temporarily life-saving in some individual patients.

(2) Its indications in early, localized Hodgkin's disease are open to question. It is not as effective as localized X-ray therapy. It is now being used in combination with intensive local X-ray therapy, but the results will take many years to evaluate.

(3) HN2 is often temporarily effective in relieving systemic symptoms of Hodgkin's disease; it may produce prompt and prolonged improvement, sometimes at doses that do not produce definite bone marrow depression.

(4) Since, in most instances, a course of HN2 is given quickly, the therapeutic response is prompt. Accordingly, HN2 is used widely in acute situations, as in pressure symptoms due to tumor involving the spinal cord or the mediastinum. Regression occurs without transient edema, which may occasionally complicate the initiation of X-ray therapy. Following a course of HN2, localized X-ray therapy is often used.

(5) All patients with Hodgkin's disease merit a trial of HN2; even in apparently far-advanced disease, striking temporary benefit may follow the first course of HN2. It has been described as effective in "radio-resistant" patients. The term radio-resistant must be defined with care. As a systemic agent, HN2 acts on all disease-bearing areas, some of which are not detectable by physical examination; thus, it may be effective in patients not responding to a local course of X-ray therapy. After a course of HN2 the patient's general condition may be improved, so that continued X-ray therapy is feasible.

(6) In patients with unexplained persistent fever, a response to a course of HN2 may support a diagnosis of lymphoma.

Presently, HN2 is used principally in hospitalized patients in whom a prompt course of adequate treatment is indicated.

Lymphosarcoma. The category of lymphosarcoma includes a number of related diseases: small-cell lymphosarcoma, leukolymphosarcoma, giant follicular lymphosarcoma, and reticulum-cell sarcoma. These diseases vary tremendously in their rate of progression and response to therapy. Some patients have shown prolonged improvement, including complete disappearance of the disease, following a course of HN2. Some patients may run a febrile course similar to that in systemic Hodgkin's disease, and HN2 may produce prompt detoxification. On the other hand, the disease may be extremely resistant and may progress despite the most vigorous administration of HN2. Thus, the response of lymphosarcoma to HN2 ranges from that seen in the most sensitive form of chronic lymphatic leukemia to the most resistant form of Hodgkin's disease, both in tolerance to the drug and in clinical improvement. In terms of consistency of response, HN2 is generally less effective in the lymphosarcomas than in Hodgkin's disease.

Mycosis fungoides. Mycosis fungoides often begins as a relatively mild and slowly progressive skin disease, but ultimately it terminates in a disseminated phase manifested as a form of lymphoma. In some cases HN2 has caused remarkable clearing of skin lesions for three to five months, but the disease has finally become refractory to this agent. In the late stages prompt regression of generalized skin nodules may occur after HN2, only to reappear within a week to ten days.[26] If treatment is continued, bone marrow depression inevitably intervenes, while the disease continues to flourish.

Multiple myeloma. Occasional relief of pain and regression of palpable masses have been associated with HN2, but this drug does not appear to have an important influence on multiple myeloma.

Polycythemia vera. Polycythemia vera has shown definite improvement on HN2. The usual duration of response to a course of the drug is in the range of three to five months, and repeated courses of treatment can be given. The duration of improvement is usually not as long, however, as that following a course of radioactive phosphorus. If maintenance management with an alkylating agent is desirable, one of the oral compounds is more conveniently used.

Carcinomas and sarcomas. The consistency of therapeutic benefit to be expected from HN2 in the various forms of carcinoma have not been determined clearly, although the indications for the use of HN2 are reasonably well established. There are several reasons for the failure to accumulate more satisfactory clinical data in these forms of cancer:

(1) The clinical results obtained with HN2 in carcinomas and sarcomas are usually poor, and the responses that may occur are usually more difficult to measure and to interpret than those in the leukemias and lymphomas. Increasing attention is now being given to the problem of defining criteria of response of the solid tumors to treatment.

(2) As newer alkylating compounds appear, organized clinical research groups expend their facilities on the evaluation of these compounds. The clinical results obtained with HN2, as an old and established form of treatment, are not often studied and reported in detail.

(3) Since a course of intravenous HN2 can be given rapidly, the drug is now being used principally in the treatment of acute problems in patients with far advanced disease. Patients in more favorable condition, who can be observed during a protracted course of treatment, are suitable for studies on the newer compounds. The comparative evaluation of HN2 and other alkylating agents must be carried out under uniform conditions of case selection and treatment schedule.

Lung cancer has been studied most systematically with HN2.[27] The first case, treated at Memorial Hospital in November 1945, is shown in FIGURE 2.

Subsequent studies have indicated that subjective relief of some degree may occur in 50 to 60 per cent of the patients, and in 30 to 40 per cent there is objective evidence of tumor regression. The response usually has been brief, in the range of three to six weeks, and second courses usually have been less effective. The most dramatic results have been obtained in patients with anaplastic carcinoma, and in those with compression of the superior vena cava. In the latter situation, 80 to 90 per cent of the patients may show prompt benefit. HN2 is often used before X-ray therapy in the treatment of nonresectable pulmonary cancer.

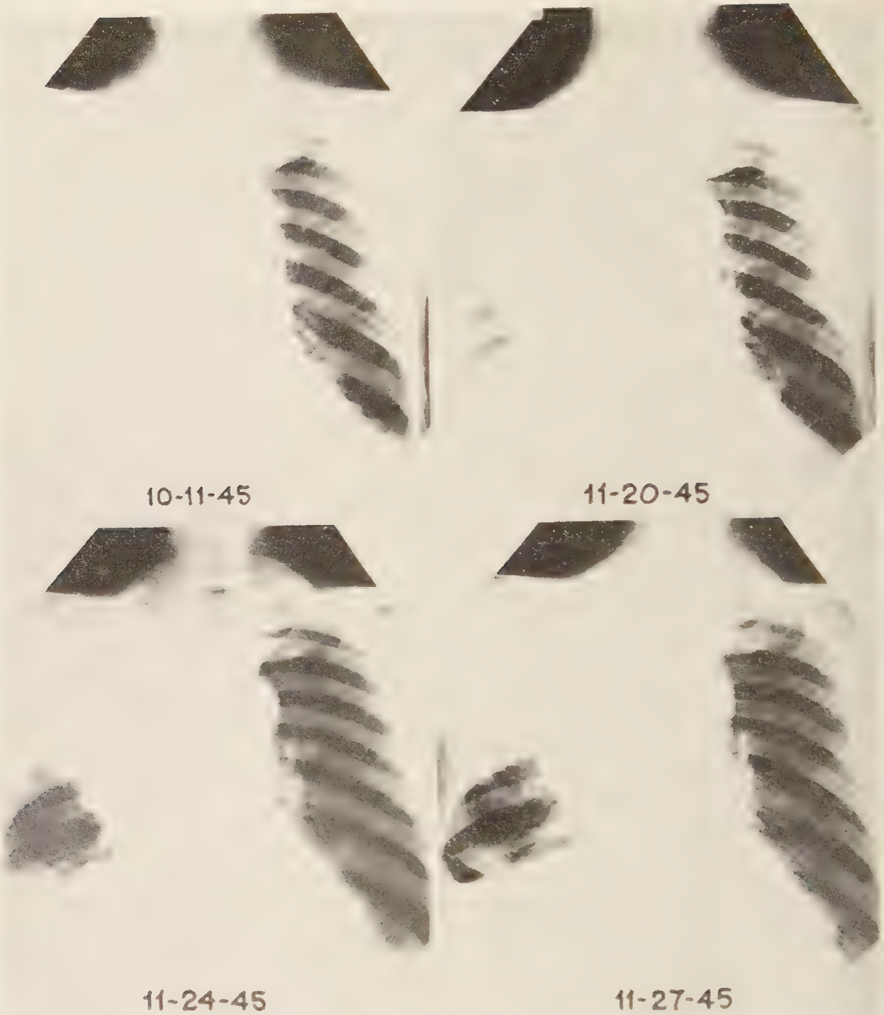

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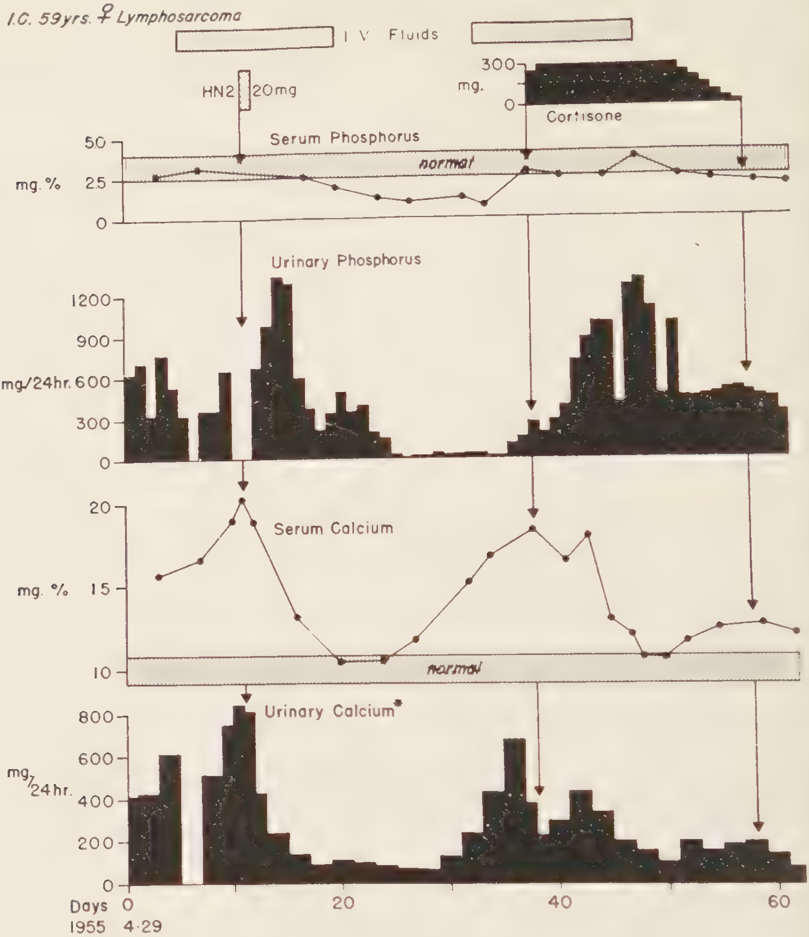
FIGURE 2. This patient, M. B., was a 60-year-old male with an established diagnosis of anaplastic carcinoma of the lung. His symptoms began in June 1945. In October, when first seen at Memorial Hospital, he had enlarged axillary nodes, peripheral edema, superior vena cava compression syndrome, and diminished vital capacity. An X-ray examination of his chest revealed an opaque right thorax (October 11, 1945). He was placed at bed rest, given a low salt diet, and his peripheral edema subsided. The X-ray examination on November 20, 1945, showed his pulmonary situation prior to treatment. On November 20 to 27 he received 6 doses of HN2 totalling 35.0 mg. (0.6 mg./kg.). Associated with this treatment there was rapid absorption of the fluid (November 24 and 27, 1945), decrease in body weight, improved dyspnea, a rise in vital capacity, subsided venous distension, and a decrease in the size of the axillary nodes. Following the response to HN2, beginning December 3, 1945, the patient received a course of X-ray therapy to the right upper chest. He continued to feel well and to do active work, but in March 1946 the disease began to generalize, and he died on April 11, 1946, with evidence of intra-abdominal metastases.

Roswit and Kaplan[28] reviewed 254 patients reported in the literature up to 1951, of whom 134 (53 per cent) obtained a favorable response.

Results in other types of cancer have not been as extensively recorded. HN2 has had little or no effect in malignant melanoma, and in most cancers of the head and neck. Certain carcinomas, which are notably radio-sensitive, such as anaplastic carcinomas of the nasopharynx and seminomas, may respond to HN2. There has been no reported series of carcinoma of the ovary treated with HN2. Sykes *et al.*[29] have reported a patient who had a good response from HN2, followed by maintenance TEM therapy; there was a decrease in an abdominal mass, and pleural effusion diminished for a five-month period. Kupfer[30] obtained regression from a retinoblastoma on HN2, and this led to interesting work in the combination of TEM and X rays in the treatment of retinoblastoma.[31] Soft part tumors may temporarily regress. Fever may be relieved in osteogenic sarcoma.

Disturbances due to cancer in certain strategic areas, such as the lungs and central nervous system, may be temporarily corrected by HN2. We have observed improvement in neurological signs from cerebral metastases, diminution in dyspnea from widespread pulmonary metastases, and disappearance of jaundice in metastatic gastric cancer after a course of HN2. Pulmonary metastases from carcinoma of the cervix have regressed. Wilms' tumor and neuroblastoma in children may temporarily regress on HN2. Impressive improvement has been obtained in two women with metastatic chorioepithelioma.[32, 33] Patients with hypercalcemia and hypercalcuria due to neoplastic disease have responded to HN2 by improvement in their serum and urinary calcium levels. W. P. L. Myers has allowed me to present two of his patients with hypercalcemia, one case due to lymphosarcoma (FIGURE 3) and the other caused by osseous metastases from a carcinoma of the kidney (FIGURE 4). The response of the lymphosarcoma was striking,[34] but alterations in the serum and urinary calcium levels suggested also that some growth restraint had been produced by HN2 in the renal carcinoma. The evidence indicated that HN2 may have some influence—transient, inconstant, and unpredictable—on carcinomas; in any large series of patients, an appreciable number of responses should be obtained, with certain forms of neoplasms being more consistently benefited.

Several attempts have been made to enhance the local effects of HN2. In patients with recurrent effusions, HN2 has been injected into the body cavities. One of the difficulties in interpreting the results is the possibility that an intravenous course of HN2 can also produce fluid resorption, as described for the pleural effusion shown in FIGURE 2. One of our best results from the intrapleural administration of HN2, however, was obtained in the first patient treated, a man with carcinoma of the lung whose pleural effusion required frequent tapping. He received intrapleural HN2 in July 1947, and his need for thoracenteses ceased. The patient survived for ten months and died of metastatic disease.[27] Another patient with carcinoma of the breast showed no further accumulation of fluid for three years after intrapleural HN2. Weisberger *et al.*[35] has reported results with intrapleural HN2 that compare favorably with those obtained with radioactive gold.



*Calcium intake approximately 200mg./day

FIGURE 3. This patient was a 59-year-old female with a diagnosis of lymphosarcoma. In the late stage of the disease she developed coma associated with a severe hypercalcemia and hypercalcuria. She received HN2 intravenously (0.3 mg./kg.) with a fall to normal in her serum calcium within a period of 10 days, and a marked reduction in the urinary calcium excretion. Hypercalcemia recurred within 10 days, and the patient again became comatose. She was started on cortisone (300 mg./day), and again there was considerable improvement in her hypercalcemia and hypercalcuria. These biochemical changes indicated the marked sensitivity to chemotherapeutic agents of the neoplastic process responsible for the hypercalcemia in this patient.

Another attempt to localize the activity of HN2 is by intra-arterial injection. This approach was developed separately by Klopp *et al.*[22] and Bierman *et al.*[36] These investigators observed regression of tumors ordinarily not responsive to HN2. Klopp used a fractionated dosage technique, so that the catheter was left in the artery for a week or more. Sullivan *et al.*[37]

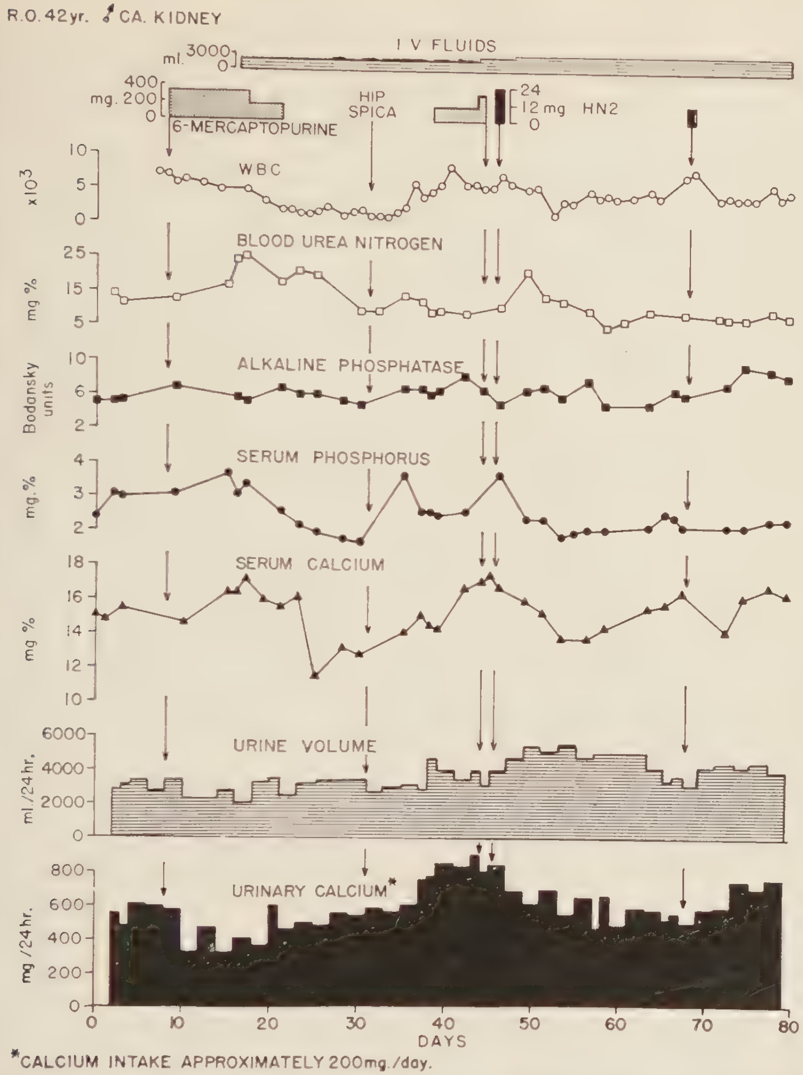


FIGURE 4. This 42 year-old male had a carcinoma of the kidney and osseous metastases with hypercalcemia and hypercalcuria. A course of 6-mercaptopurine resulted in a slight transient fall in the elevated serum calcium and increased urinary calcium excretion. A similar result was obtained with a course of HN2. These biochemical observations suggested that 6-mercaptopurine and HN2 produced a brief interruption in the progression of the disease. It is apparent that the degree of improvement in calcium metabolism produced in this patient by HN2 was considerably less than the response that occurred in the patient with lymphosarcoma (FIGURE 3).

developed a simplified, rapid treatment technique involving the puncture of the artery and the introduction of a polyethylene catheter into it.

Cromer *et al.*[38] reported some degree of improvement in 12 of 18 patients with carcinoma of the vagina and cervix treated by fractionated intra-arterial HN2. Sullivan *et al.*[37] observed regression in pelvic tumors in 4 of 8 cases. Krakoff[39] treated 20 cases of cancer involving the pelvic structures. He observed some degree of response in 3 of 6 cases of carcinoma of the cervix, in 5 of 7 cases of ovarian cancer, in 2 of 2 cases of uterine cancer, and in a single patient with osteogenic sarcoma. Four patients with carcinoma of the large bowel failed to respond. Improvement, albeit temporary, consisted of a reduction in the size of a pelvic mass, relief of uremia due to ureteral obstruction, decrease in intestinal obstruction, and the relief of pain.

In conclusion, the following points can be stated. HN2 is effective, within the limitations noted, in some cases and at some stages of Hodgkin's disease, lymphosarcoma, and related conditions; in chronic myelocytic leukemia and chronic lymphocytic leukemia; and in polycythemia vera. It can cause relief of local symptoms and tumor regression in certain strategically placed and rapidly growing tumors; for example, in the lung, in the chest, and in the central nervous system. Certain radio-sensitive tumors, such as seminomas, and carcinomas of the nasopharynx and of the ovary, may respond temporarily. The drug has produced tumor regression in chorioepithelioma in the female, in carcinoma of the cervix, and in breast cancer; the duration of the therapeutic response is related largely to the natural rate of progression of the disease. It has relieved bone pain and fever in osteogenic sarcoma. We believe that, as far as cancer is concerned, HN2 sensitivity parallels radio sensitivity.

The intrapleural and intra-arterial injection of HN2 enhances its local effect at the site of injection.

HN2 has certain advantages in practice; a course of treatment is usually given within a short time; the treatment and the evidences of a therapeutic response, when it occurs, will be close together. Dosage of HN2 is well established, and it can be given safely. The disadvantages of the drug, as compared to other alkylating agents, are the need for intravenous injection, and the nausea and vomiting that make treatment intermittent and often necessitates hospitalization. Prolonged maintenance therapy, therefore, is accomplished more easily with the oral alkylating agents.

We may ask the question: if HN2 were given at an equivalent biological dose and time schedule as the other polyfunctional alkylating agents (to be discussed), would we obtain an identical spectrum and degree of therapeutic activity in relation to bone marrow depression in various forms of cancer? The theme today is comparison, and we must face the problem. Do all these agents act alike, or is there such a thing as inherent specificity for certain tumors or normal tissues among the different alkylating agents?

References

1. Gilman, A., L. S. Goodman, F. S. Philips & T. Dougherty. 1943. Unpublished.
2. Rhoads, C. P. 1946. Nitrogen mustards in treatment of neoplastic disease: officia statement. J. Am. Med. Assoc. **131**: 656–658.

3. GOODMAN, L. S., M. M. WINTROBE, W. DAMESHEK, M. J. GOODMAN, A. GILMAN & M. T. MCLENNAN. 1946. Nitrogen mustard therapy. J. Am. Med. Assoc. **132**: 126–132.
4. GOODMAN, L. S., M. M. WINTROBE, M. T. MCLENNAN, M. DAMESHEK, M. J. GOODMAN & A. GILMAN. 1947. Use of methyl-bis(β-chloroethyl)amine hydrochloride and tris(β-chloroethyl)amine hydrochloride (nitrogen mustards) in the therapy of Hodgkin's disease, lymphosarcoma, leukemia and certain allied and miscellaneous disorders. *In* Approaches to Tumor Chemotherapy. : 338–346. Am. Assoc. Advance. Sci. Washington, D. C.
5. JACOBSON, L. O., C. L. SPURR, E. S. GUZMAN-BARRON, T. SMITH, C. LUSHBAUGH & G. F. DICK. 1946. Studies on effect of methyl-bis(β-chloroethyl)amine hydrochloride on neoplastic diseases and allied disorders of hemopoietic system. J. Am. Med. Assoc. **132**: 263–271.
6. SPURR, C. L., L. O. JACOBSON, T. R. SMITH & E. S. GUZMAN-BARRON. 1947. The clinical application of methyl-bis(β-chloroethyl)amine hydrochloride to the treatment of lymphomas and allied dyscrasias. *In* Approaches to Tumor Chemotherapy. : 306–318. Am. Assoc. Advance. Sci. Washington, D. C.
7. KARNOFSKY, D. A., L. F. CRAVER, C. P. RHOADS & J. C. ABELS. 1947. An evaluation of methyl-bis(β-chloroethyl)amine hydrochloride and tris(β-chloroethyl)amine hydrochloride (nitrogen mustards) in the treatment of lymphomas, leukemia and allied diseases. *In* Approaches to Tumor Chemotherapy. : 319–337. Am. Assoc. Advance. Sci. Washington, D. C.
8. WILKINSON, J. F. & F. FLETCHER. 1947. Effect of β-chloroethylamine hydrochlorides in leukemia, Hodgkin's disease and polycythaemia vera. Lancet. **2**: 540–545.
9. RHOADS, C. P. 1947. Report on a cooperative study of nitrogen mustard (HN2) therapy of neoplastic disease. Trans. Assoc. Am. Physicians. **60**: 110–117.
10. KARNOFSKY, D. A. 1950. Nitrogen mustards in the treatment of neoplastic disease. Advances in Internal Med. **4**: 1–75.
11. KLOPP, C. T. & J. C. BATEMAN. 1954. Some aspects of the clinical use of nitrogen mustards. Advances in Cancer Research. **2**: 255–279. J. P. Greenstein and A. Haddow, Eds.
12. CRAVER, L. F. 1948. The nitrogen mustards: clinical use. Radiology. **50**: 486–493.
13. ZANES, R. P., C. A. DOAN & H. A. HOSTER. 1948. Studies in Hodgkin's syndrome. VII. Nitrogen mustard therapy. J. Lab. Clin. Med. **33**: 1002–1018.
14. BLOCK, M., C. L. SPURR, L. O. JACOBSON & T. R. SMITH. 1948. Histopathologic effects of nitrogen mustard therapy upon normal and neoplastic hematopoietic tissues. Am. J. Clin. Pathol. **18**: 671–689.
15. WINTROBE, M. M. & C. M. HUGULEY, Jr. 1948. Nitrogen mustard therapy for Hodgkin's disease, lymphosarcoma, the leukemias, and other disorders. Cancer. **1**: 357–381.
16. ARIEL, I. M. & L. KANTER. 1949. Nitrogen mustard therapy. Clinical studies on the effects of methyl-bis(β-chloroethyl)amine hydrochloride upon various types of neoplastic disease. Am. J. Surgery. **77**: 509–521.
17. BIERMAN, H. R., M. B. SHIMKIN, S. R. METTIER, J. WEAVER, S. C. BERRY & S. P. WISE, III. 1949. Methyl-bis(β-chloroethyl)amine in large doses in the treatment of neoplastic diseases. Calif. Med. **71**: 117–125.
18. DAMESHEK, W., L. WEISFUSE & T. STEIN. 1949. Nitrogen mustard therapy in Hodgkin's disease: analysis of fifty consecutive cases. Blood. **4**: 338–379.
19. ERF, L. A. & R. D. BAUER. 1949. The clinical effect of nitrogen mustard on Hodgkin's disease. Am. J. Clin. Pathol. **19**: 372–380.
20. SPURR, C. L., T. R. SMITH, M. BLOCK & L. O. JACOBSON. 1950. The role of nitrogen mustard therapy in the treatment of lymphomas and leukemias. Am. J. Med. **8**: 710–723.
21. JAGER, V., M. M. WINTROBE, A. M. GINZLER & S. GORDON. 1943. Oral ingestion of tris(β-chloroethyl)amine hydrochloride by humans. Rept. No. 1. Med. Research Lab., Medical Div., Army Medical Center. Washington, D. C.
22. KLOPP, C. T., T. C. ALFORD, J. BATEMAN, G. N. BERRY & T. WINSHIP. 1950. Fractionated intra-arterial cancer chemotherapy with methylbis-amine hydrochloride; a preliminary report. Ann. Surg. **132**: 811–832.

23. Klima, R. & R. Hornisher. 1953. Nitrogen mustard therapy of proliferative blood diseases: method, experience and clinical application in cancer therapy. Wien. med. Wochschr. **103**: 11–14.
24. Burchenal, J. H., W. P. L. Myers, L. F. Craver & D. A. Karnofsky. 1949. The nitrogen mustards in the treatment of leukemia. Cancer. **2**: 1–17.
25. Gellhorn, A. & V. P. Collins. 1951. A quantitative evaluation of the contribution of nitrogen mustard to the therapeutic management of Hodgkin's disease. Ann. Internal Med. **35**: 1250–1259.
26. Henstell, H. H., J. N. Tober & B. A. Newman. 1947. The influence of nitrogen mustard on mycosis fungoides; observations relating its effect to the reticulo-endothelial system. Blood. **2**: 564–577.
27. Karnofsky, D. A., W. H. Abelmann, L. F. Craver & J. H. Burchenal. 1949. The use of the nitrogen mustards in the palliative treatment of carcinoma, with particular reference to bronchogenic carcinoma. Cancer. **1**: 634–656.
28. Roswit, B. & G. Kaplan. 1951. Nitrogen mustard as an adjunct to radiation in the management of bronchogenic cancer. Radiology. **57**: 384–394.
29. Sykes, M. P., R. W. Rundles, V. K. Pierce & D. A. Karnofsky. 1955. Triethylene melamine in the management of far advanced ovarian cancer. Surgery. **101**: 133–140.
30. Kupfer, C. 1953. Retinoblastoma treated with intravenous nitrogen mustard. Am. J. Ophthalmol. **36**: 1721–1723.
31. Hyman, G. A. & A. B. Reese. 1956. Combination therapy of retinoblastoma with triethylene melamine and radiotherapy. J. Am. Med. Assoc. **162**: 1368–1373.
32. Anderson, H. E., J. D. Bisgard & A. M. Greene. 1954. Metastatic chorioepithelioma of lung with nitrogen mustard therapy: preliminary report. A. M. A. Arch. Surg. **68**: 829–837.
33. Karnofsky, D. A., W. P. L. Myers & R. Phillips. 1955. Treatment of the inoperable pulmonary cancer, preliminary and metastatic. Am. J. Surgery. **89**: 526–537.
34. Myers, W. P. L. 1956. Clinical aspects and management of hypercalcemia. Med. Clin. N. Am. (Memorial Center Number). **40**: 871–885.
35. Weisberger, A. S., B. Levine & J. P. Storaasli. 1955. Use of nitrogen mustard in treatment of serous effusions of neoplastic origin. J. Am. Med. Assoc. **159**: 1704–1707.
36. Bierman, H. R., K. H. Kelly, R. L. Byron, Jr., K. S. Dod, & M. B. Shimkin. 1951. Studies on the blood supply of tumors in man. II. Intra-arterial nitrogen mustard therapy of cutaneous lesions. J. Natl. Cancer Inst. **11**: 891–905.
37. Sullivan, R. D., R. Jones, Jr., T. G. Schnabel, Jr. & J. M. Shorey. 1953. The treatment of human cancer with intra-arterial nitrogen mustard [methyl-bis(2-chloroethyl)amine hydrochloride] utilizing a simplified catheter technique. Cancer. **6**: 121–134.
38. Cromer, J. K., J. C. Bateman, G. N. Berry, J. M. Kennelly, C. T. Klopp & L. I. Platt. 1952. Use of intra-arterial nitrogen mustard therapy in the treatment of cervical and vaginal cancer. Am. J. Obstet. Gynecol. **63**: 538–548.
39. Krakoff, I. Intra-arterial nitrogen mustard in the treatment of pelvic cancer. In press.

CLINICAL EVALUATION OF CB 1348 IN MALIGNANT LYMPHOMA AND RELATED DISEASES

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The introduction of the aliphatic nitrogen mustards as compounds for the treatment of malignant disease was a significant advance in the chemotherapy of cancer. The beneficial effect of these cytotoxic agents prompted a systematic search for compounds of greater selectivity of site of action and fewer toxic side effects. The synthesis of a series of water-soluble aromatic nitrogen mustards by Everett *et al.*[1] included *p*-(di-2-chloroethylamino)-phenylbutyric acid (CB 1348, chlorambucil, Leukeran), which was selected for clinical trial because of its marked inhibitory effect in the rat on the transplanted Walker tumor 256.[2] Subsequently we reported our clinical results with this compound in 62 cases of malignant lymphoma.[3]

Altman *et al.*[4] found CB 1348 to be an effective and well tolerated agent in the treatment of chronic lymphocytic leukemia. The compound was reported by Bouroncle *et al.*[5] to be of value in Hodgkin's disease as a supplement to radiation. These investigators obtained excellent remissions in 6 and improvement in 9 in a total of 24 patients. The relative safety of CB 1348 was confirmed by Ultmann *et al.*,[6] who obtained satisfactory responses in chronic lymphocytic leukemia, although their results in Hodgkin's disease and lymphosarcoma were not impressive. Hall *et al.*[7] reported that CB 1348 was an effective agent in chronic lymphocytic leukemia and lymphosarcoma, but was of less value in Hodgkin's disease and mycosis fungoides.

In this further report on our experience with CB 1348 we include those cases previously reported from the Chester Beatty Research Institute and Royal Marsden Hospital and from the Winnipeg General Hospital. The series now includes 109 cases treated over a 4-year period. There are 35 cases of Hodgkin's disease, 27 of chronic lymphocytic leukemia, 20 of aleukemic lymphocytic lymphoma, 12 of follicular lymphoma, 13 of reticulum cell sarcoma, and 2 of acute monocytic leukemia.

Administration and Dosage

The CB 1348 was usually given orally, the dose ranging from 2 to 40 mg. per day. Most cases on oral therapy received 0.1 to 0.2 mg. kg./day (6 to 12 mg./day in the average patient). When the drug was effective, improvement was noted in 3 to 4 weeks. Those cases on adequate dosage in which no response was observed within 6 weeks did not respond to increased dosage or to continuation of therapy for longer periods of time. The usual course of treatment was 4 to 8 weeks. Treatment was usually stopped when a satisfactory remission was obtained, but a few of our early cases received maintenance therapy over long periods of time. In those cases showing continuing improvement and a blood picture that remained satisfactory, treatment was often continued until complete regression of lymphadenopathy and splenomegaly had been achieved.

Thirteen patients received the sodium salt intravenously. The dose was 10 to 40 mg. per day for 3 to 4 days, repeated at 2- to 3-week intervals if the leukocytes and platelets remained at a satisfactory level and further treatment was indicated. The injections were well tolerated and did not damage the veins.

Results

The results are summarized in FIGURE 1 and are depicted graphically for each disease in FIGURES 2 to 6. The clinical response is classified as "benefit," "some effect," and "no effect." "Benefit" denotes improvement of real value, with subsidence of all symptoms and return to normal activity for at least 6 months. "Some effect" denotes subjective and objective improvement of real value, but of less than 6 months' duration; some of these eventually will enter the "benefit" group.

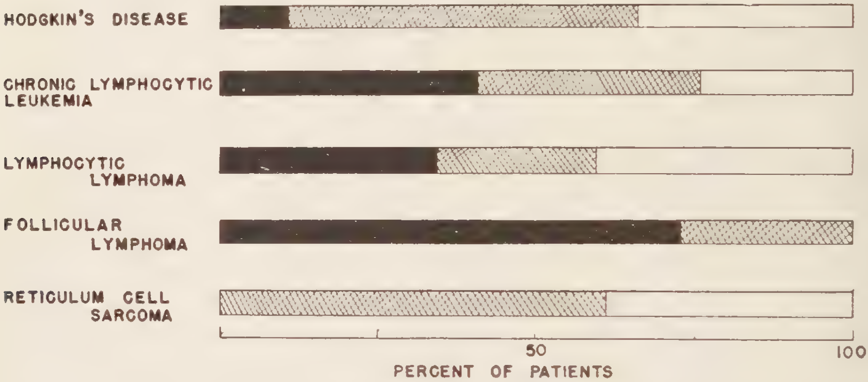

FIGURE 1. Clinical response. The number of patients in each group is: Hodgkin's disease, 35; chronic lymphocytic leukemia, 27; lymphocytic lymphoma, 20; follicular lymphoma, 12; and reticulum cell sarcoma, 13.

In the accompanying figures, the total clinical response is noted with ■ - indicating "benefit"; ▩ "some effect"; and □ - "no effect." The beneficial effect of previous and subsequent therapy is designated by ▨ within a bar indicating the number of patients treated. Regression of lymph nodes, spleen, and liver; subsidence of fever, and rise in hemoglobin of more than 2 gm. per cent is shown as ▨ within the bar, indicating, respectively, the number of patients with lymphadenopathy, splenomegaly, hepatomegaly, fever, or anemia. Neutropenia refers to a total neutrophil count of less than 1000 per cu. mm. and thrombocytopenia to less than 100,000 platelets per cu. mm.

Hodgkin's Disease (*FIGURE 2*)

In this group of 35 patients, 4 responses are classed as "benefit" and 21 as "some effect." Seven patients were treated with intravenous therapy. Of the 35 patients treated, 29 had had previous therapy—23 with radiation and 3 each with mechlorethamine hydrochloride (HN2) and triethylene melamine (TEM). Twenty had previously responded to X ray and, of these, 3 showed subsequent benefit and 9 some effect from CB 1348. Two cases that responded poorly to X ray obtained some effect from the drug. Of the patients that had responded previously to HN2 or TEM, 2 of the 3 in each group derived some effect from later CB 1348 therapy. The response of the fever, lymphadenopathy, splenomegaly, and hepatomegaly and accompanying anemia is shown in FIGURE 2. The duration of improvement in those cases that responded was from 3 weeks to 15 months, but in most cases the remissions lasted 4 to 10 weeks. Nine patients in relapse were again treated with CB 1348. Six responded and are classed as "some effect." Once relapse occurred, response to other therapy was poor, although 2 of 6 subsequently responded to steroids.

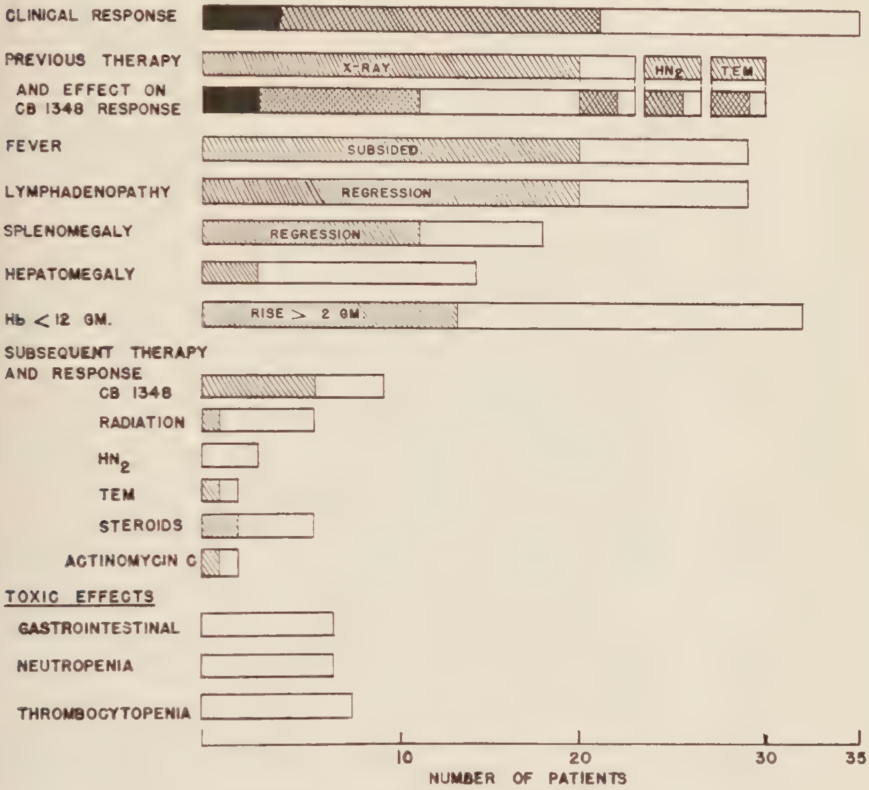

FIGURE 2. Hodgkin's disease: 35 patients.

Gastrointestinal side effects occurred in 7 cases. Five had transient nausea and vomiting, 1 had retrosternal burning, and 1 had diarrhea. All were receiving 20 to 40 mg. per day. Seven had transient neutropenia, and 4 of these had also an associated thrombocytopenia. Six of these cases had received in excess of a total dosage of 6.0 mg. kg. There were 4 other cases of transient thrombocytopenia.

The results in Hodgkin's disease are only fair. There are only 4 cases with sustained remission in the group. Although 21 are classed as "some effect," the response was often transient. The more recent cases have been given intravenous CB 1348, usually 40 mg. daily for 3 to 4 days and then repeated in 3 to 4 weeks if the leukocytes and thrombocytes are at a satisfactory level. This regimen may prove to be somewhat better than that previously employed.

Chronic Lymphocytic Leukemia (FIGURE 3)

This group consists of 27 patients. Twelve patients are classed as "benefit" and 9 more as "some effect." Four of this second group are doing well and probably will enter the benefit group within a few months. Eleven patients received previous X-ray therapy, and 1 received HN2. Of the 7 patients that previously responded to radiation, 1 derived benefit from CB 1348 and 5 obtained some effect. Two of the 4 patients who responded poorly to X ray subsequently responded to CB 1348 and are classed as "some effect." The patient who initially responded well to HN2 also did well on CB 1348. These patients have remained well for periods of from 5 months

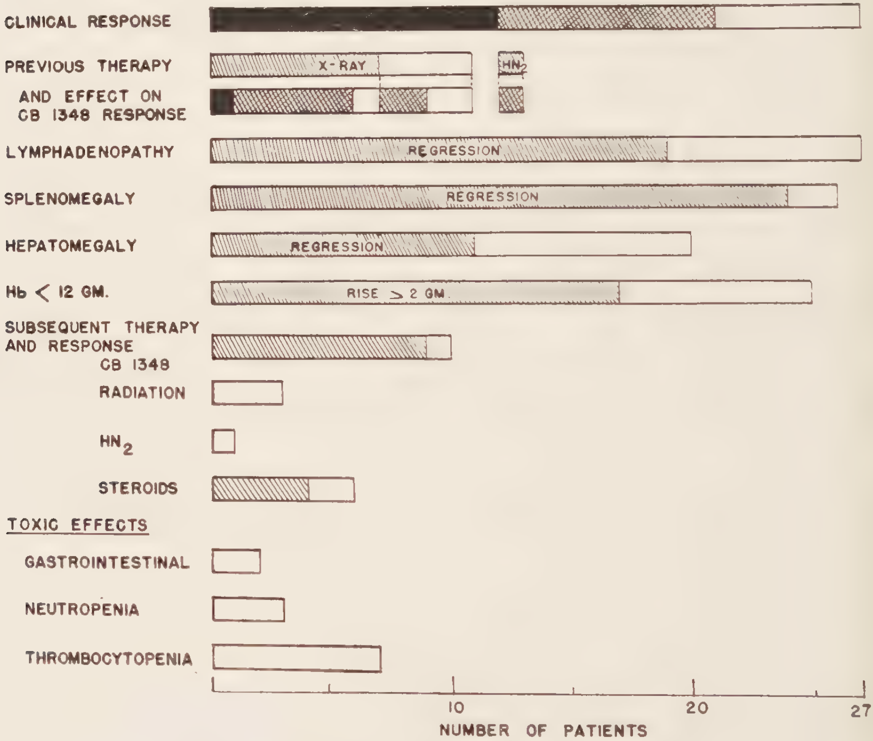

FIGURE 3. Chronic lymphocytic leukemia: 27 patients.

to $3\frac{1}{2}$ years. Ten patients have had more than one course of treatment, 8 have had 1 subsequent remission, and 1 patient has responded twice. The only other subsequent therapy of value in this group is the steroids, which were effective in 4 of 6 cases. Neutropenia and thrombocytopenia occurred at lower total dosage levels in this group than in the other lymphomas. All cases developing neutropenia and 6 of the 7 with thrombocytopenia did so on a total dose of less than 6 mg./kg.

The results in this group are good. Several patients have been maintained in good health with CB 1348 alone for periods up to $3\frac{1}{2}$ years. Favorable effects included symptomatic relief; weight gain; regression of lymphadenopathy, splenomegaly, and hepatomegaly; an increase in neutrophils; and a reduction in lymphocytosis. A sustained rise in hemoglobin was observed in 17 cases, with arrest of active hemolysis in 4 instances.

Aleukemic Lymphocytic Lymphoma (FIGURE 4)

This represents a group of 20 patients that clinically, except for the absence of circulating lymphocytosis, closely resembles the preceding group. Most patients had lymphadenopathy, and many had enlargement of the liver and spleen. The histology of the lymph nodes was similar to that of the chronic

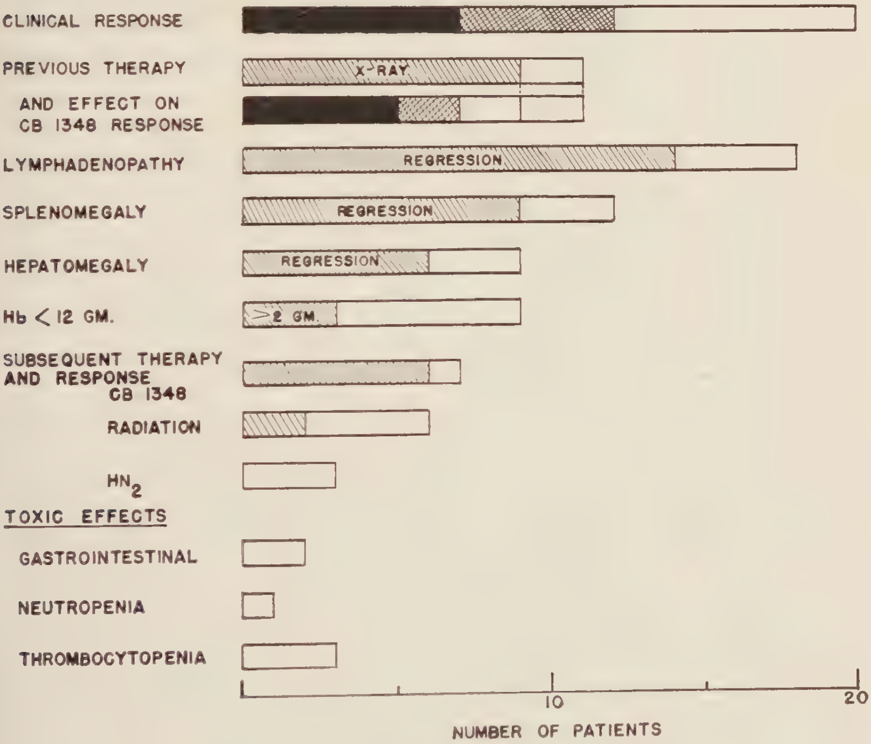

FIGURE 4. Lymphocytic lymphoma: 20 patients.

lymphocytic leukemia group. The bone marrow showed lymphocytic infiltration in 13 cases. We have not subdivided this group into the so-called lymphoblastic lymphomas and lymphocytic lymphomas.

Seven of these patients are classified as "benefit" and 5 more as "some effect"; no response was obtained in 8. Eleven had had previous X-ray therapy, to which 9 had responded. Of those previously responding to X ray, 5 derived benefit and 2 some effect from CB 1348. The 2 who responded poorly to X ray also responded poorly to CB 1348. The improvement on therapy ranged from 3 months to 3 years. Seven patients were treated following relapse; 5 obtained one subsequent remission and 1 achieved 2 remissions. A response to radiation occurred in 2 of 6 patients receiving X ray following CB 1348 relapse. The toxic effects in this group were minor.

Seven of these patients derived significant benefit from CB 1348. Three of the 5 classified as "some effect" will probably soon move into the "benefit" class. The drug would seem to be of real value in the treatment of this group.

Follicular Lymphoma (FIGURE 5)

There are 12 patients in this group, 9 classified as "benefit" and 3 as "some effect." Of these last 3 cases, 1 died of a coronary occlusion in the

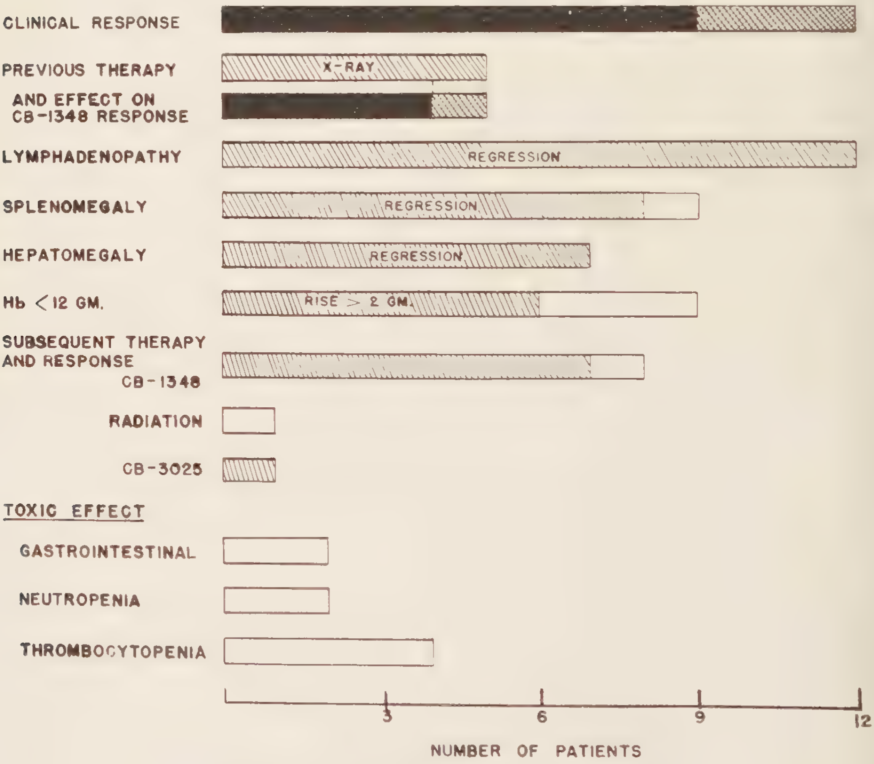

FIGURE 5. Follicular lymphoma: 12 patients.

third month of her remission and another is doing well in her fifth month of remission and may soon enter the "benefit" category. Five had been treated previously with X ray, and 4 of these subsequently showed an excellent response to CB 1348. Following relapse in 8 patients, 7 responded again to CB 1348. Three patients have had a second, 1 a third, 1 a fourth, and 2 a fifth good response to subsequent CB 1348 therapy. The responses have varied from 3 to 27 months, and 6 are of more than 1 year's duration. The toxic effects were minimal, although depression of platelets and granulocytes occurred in those patients with bone-marrow invasion. The two patients who developed neutropenia had received a total dosage in excess of 7.5 mg./kg.

The results in this group are good, with relief of symptoms and remissions of long duration.

Reticulum Cell Sarcoma (FIGURE 6)

This group of 13 patients had widely disseminated disease, many with involvement of bone, skin infiltrations, and ulceration. The course was rapidly progressive. Six patients had received previous X-ray therapy and

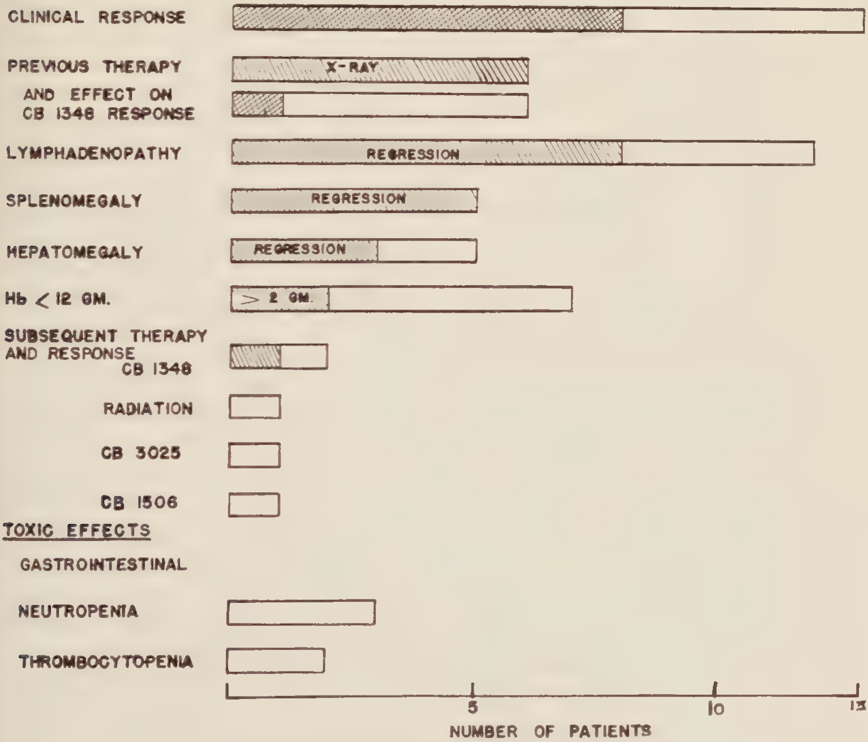

FIGURE 6. Reticulum cell sarcoma: 13 patients.

all had derived some benefit. Only 1 of these responded subsequently to CB 1348. All the previously untreated cases made some response to CB 1348 and have been classed as "some effect." Although the response was often dramatic, it was of short duration, and relapse usually occurred within 3 months. Of 2 patients who relapsed and were treated subsequently with CB 1348, 1 had a brief further response. No other therapy was of benefit in this group in relapse. Three patients developed neutropenia and 1 died of agranulocytosis; this last patient had received 586 mg. in a 3-week period. Two of these neutropenic cases also had thrombocytopenia.

Acute Monocytic Leukemia

Two cases of acute monocytic leukemia have been treated with good temporary response:

Case I. A 40-year-old female nurse presented with fever, weakness, and a petechial rash over her trunk and thighs. There was hypertrophy of the gums, skin infiltrates, lymphadenopathy, splenomegaly, and hepatomegaly. WBC—140,000 cu. mm., with 78 per cent immature monocytes; Hb 11.5 gm.; platelets—350,000 cu. mm. Bone marrow examination confirmed the diagnosis. She received 105 mg. of CB 1348 in 9 days (1.7 mg. kg.). As the white blood count had fallen to 15,000, the dose was reduced to 2 mg. daily for the next 4 weeks. At the termination of therapy the nodes, liver, and spleen had completely regressed, as had the skin nodules and the gum hypertrophy. The petechial rash disappeared and, 1 month after stopping the drug, the patient was asymptomatic. At that time the WBC was 4100/cu. mm., with a normal differential; Hb 11.2 gm.; platelets 225,000 cu. mm. She relapsed 3 months after therapy was discontinued and failed to respond to a second course of CB 1348.

Case II. A 41-year-old farmer presented with weakness, tiredness, and nose-bleeds of 2 months' duration. There was generalized purpura, lymphadenopathy, gum hypertrophy, and ulcerations of the buccal mucosa. WBC 15,200 cu. mm., with 37 per cent immature monocytes; Hb 11.4 gm.; platelets 48,000 cu. mm. The bone marrow was hypercellular, with a preponderance of immature monocytes. The patient was treated with 6-mercaptopurine for 7 weeks, during which time the ulcers in his mouth healed and the white blood cell count fell to 4500 cu. mm., with 10 per cent monoblasts. However, he had developed skin infiltrations on his forehead, and the lymphadenopathy had increased. He was started on CB 1348 (0.06 mg. kg. day). Improvement was noted in 2 weeks and, at the end of 6 weeks of therapy, the skin infiltrations and gum hypertrophy had regressed completely, the lymphadenopathy was less, and the purpura had disappeared. The white blood count was 16,000 cu. mm., with 31 per cent monoblasts; Hb 13.0 gm.; platelets 50,000 cu. mm. The patient remained subjectively well for the next 6 weeks and then relapsed. He did not respond to further CB 1348 therapy. The duration of improvement on CB 1348 was 10 weeks.

In both cases, regression of skin lesions, gum hypertrophy, and decrease in lymphadenopathy, splenomegaly, and hepatomegaly occurred, accompanied by a rise in hemoglobin. Both remissions were of real value, but of short duration.

Discussion

In our first report on the clinical trial of CB 1348 we concluded that it was an agent of definite value in the treatment of chronic lymphocytic leukemia, lymphocytic lymphoma, and follicular lymphoma. The results in Hodgkin's disease were rather disappointing in that, although a number of remissions were obtained, for the most part they were of short duration. CB 1348 caused little subjective discomfort apart from mild gastrointestinal disturbances in a few patients, and it depressed hematopoietic tissue less than did HN2 and TEM. The present study confirms most of our previous impressions.

The diseases responding best to CB 1348 are those characterized primarily by abnormal lymphocytic proliferation. This is in keeping with the experimental work of Bollag[2] and Elson,[3] who found this agent to produce marked lymphocyte destruction. This relative specificity allows destruction of lymphocytes at dose levels with lesser effects on other cells, which is reflected in the much better response of chronic lymphocytic leukemia, lymphocytic lymphoma, and follicular lymphoma than of reticulum cell sarcoma and the granulomatous lesion of Hodgkin's disease.

The specificity of CB 1348 is only one of degree, as seen from the thrombocytopenia and neutropenia that result in some cases. It is to be stressed that, while CB 1348 is less toxic to granulocyte and thrombocyte production than HN2 and TEM, it can produce marrow aplasia, and the therapy must be controlled by blood counts done at not less than 3-week intervals while the patient is on active treatment. Neutropenia is most likely to occur in those cases with bone-marrow invasion by lymphomatous tissue, as is generally true for the other nitrogen mustard compounds in present use. Neutropenia has been noted with more frequency in those cases that have received CB 1348 in excess of 6 mg./kg. of body weight. This is only a rough guide, however, as many patients receiving more than this amount (as much as 23 mg./kg.) maintain normal granulocyte counts, and neutropenia has been observed in patients who received as little as 4.5 mg./kg. The thrombocytopenia bears less relationship to the total dosage than does the neutropenia. The platelets and neutrophils usually rise rapidly following discontinuation of the drug or reduction in dosage. However, delayed neutropenia has been observed to develop as long as two weeks after termination of treatment with CB 1348. That it is less toxic in this regard than HN2 or TEM makes it valuable in the treatment of those patients presenting with thrombocytopenia or neutropenia, which are relatively common in the lymphocytic lymphoma and chronic lymphocytic leukemia groups.

The response to CB 1348 again was found to be best in those patients with chronic lymphocytic leukemia, lymphocytic lymphoma, and giant follicular lymphoma. Because of its low toxicity, it is probably the chemotherapeutic agent of choice in these diseases at this time. Subjective improvement was accompanied by regression of lymphadenopathy, splenomegaly, and hepatomegaly, and a significant rise in hemoglobin occurred in most cases with an associated anemia. Previous therapy had little influence on the results. In most cases, one or more remissions were obtained on subsequent treatment with CB 1348 following relapse. The agents of most value in the chronic lymphocytic leukemia group following lack of response or relapse while on CB 1348 were the steroids. This was particularly true in those cases with a complicating acquired hemolytic anemia.

The response in Hodgkin's disease is not as good as in those groups characterized primarily by abnormal lymphocyte proliferation, and it would seem that HN2 is a more effective agent in this disease. Although regression of lymph nodes, spleen, and liver, rise in hemoglobin, and subsidence of fever were frequently observed, the effects were, for the most part, of short duration. Previous therapy seems to have little influence on the response to CB 1348. Better results than those presented in this series have been reported.[5] Nabarro,[10] in a series of 23 cases treated with CB 1348, noted benefit in 6 and some effect in an additional 6 patients. This difference may indicate some selection, as our cases are drawn from a specialized cancer hospital and cancer therapy unit rather than from a general hospital population.

As a group, the patients with reticulum cell sarcoma did poorly. Remissions were short, and the course of the disease was rapid, although lymph nodes regressed and liver and spleen decreased in size. Only one of the patients in this group who had had previous treatment responded to CB 1348, possibly because the drug was given later in the natural course of the disease. We have not been able to predict in advance, either by clinical findings, or by histological or cytological appearance of biopsy material, which patients in each lymphoma group will respond to CB 1348.

The results in acute monocytic leukemia are of interest. This is a most difficult disease to treat, and even short remissions are difficult to obtain. Bouroncle *et al.*[5] treated 10 patients with acute monocytic leukemia and observed good results in 1 and some effect in 2 other cases. A preliminary communication by Shanbrom *et al.*[11] indicates that distinct clinical and hematological improvement was noted in their cases of acute monocytic leukemia. It would seem that CB 1348 merits further trial in this disease.

Summary

One hundred and seven patients with malignant lymphomas have been treated with CB 1348 during the past 4 years 35 with Hodgkin's disease, 13 with reticulum cell sarcoma, 12 with giant follicular lymphoma, 27 with chronic lymphocytic leukemia, and 20 with lymphocytic lymphoma. Excellent remissions of at least 6 months' duration were obtained in 4 cases of Hodgkin's disease, 9 of giant follicular lymphoma, 12 of chronic lymphocytic leukemia, and 7 of lymphocytic lymphoma. Some beneficial effect was obtained in other cases, including 21 cases of Hodgkin's disease, 8 of reticulum cell sarcoma, 3 of follicular lymphoma, 9 of chronic lymphocytic leukemia, and 5 of lymphocytic lymphoma. In addition, some beneficial effects were observed in 2 cases of acute monocytic leukemia.

The drug was well tolerated and the gastrointestinal side effects were minor. Although neutropenia and thrombocytopenia did occur, it is relatively less toxic to hematopoietic tissue than are other cytotoxic agents in common use. Its low toxicity and the relatively good response in the giant follicular lymphoma, chronic lymphocytic leukemia, and lymphocytic lymphoma groups recommend it as a valuable agent in the treatment of these diseases.

References

1. Everett, J. L., J. R. Roberts & W. C. J. Ross. 1953. J. Chem. Soc. : 2386.
2. Haddow, A. 1952. British Empire Cancer Campaign. 30th Ann. Rept. **30**: 25.
3. Galton, D. A. G., L. G. Israels, J. D. N. Nabarro & M. Till. 1955. Brit. Med. J. **2**: 1172.
4. Altman, S. J., A. Haut, G. E. Cartwright & M. M. Wintrobe. 1956. Cancer. **9**: 512.
5. Bouroncle, B. A., C. A. Doan, B. K. Wiseman & W. J. Frajola. 1956. A.M.A. Arch. Internal Med. **97**: 703.
6. Ultmann, J. E., G. A. Hyman & A. Gellhorn. 1956. J. Am. Med. Assoc. **162**: 178.
7. Hall, B. E., L. P. White, J. D. Smyth, M. Tyan, F. M. Willett, T. V. Feichtmeir & E. M. Farber. 1956. Clin. Research Proc. **4**: 221.
8. Bollag, W. 1953. Schweiz. med. Wochschr. **83**: 1027.
9. Elson, L. A. 1955. Brit. J. Haematol. **1**: 104.
10. Nabarro, J. D. N. Personal communication.
11. Shanbrom, E., A. G. Knudson, Jr. & S. Rappaport. 1957. Clin. Research Proc. **5**: 31.

SUMMARY OF RESULTS OBTAINED WITH TEM

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Late in 1949[1-3] triethylene melamine (TEM*) was found to have nitrogen mustard-like effects on experimental animal tumors and leukemia. Since the compound could be given by mouth without producing serious gastrointestinal or other side reactions,[4] clinical studies were of particular interest. Karnofsky and his collaborators[5] soon demonstrated that TEM had therapeutic properties in human neoplastic diseases comparable to those of the nitrogen mustard compounds. The oral route of administration seemed to represent an important advance in practical therapy. Their findings have been confirmed amply in many clinics in different parts of the world.[6-19]

The chief deterrent to the general clinical use of TEM has been variable dosage effects in different patients. Even when given in the fasting state with alkali, as suggested by Gellhorn *et al.*,[6] absorption and or sensitivity to the chemical has varied greatly. About 1 patient in 10 will require 15 to 25 mg. or more of the chemical initially to obtain a therapeutic effect, and then 2.5 to 5 mg. per week for maintenance. About 1 in 20, on the other hand, can take only two 2.5-mg. doses initially and only 0.5 to 1.0 mg. every 7 to 14 days for maintenance without risking serious depression of bone marrow function. The dose of TEM that is both safe and therapeutically effective must be determined individually, regardless of such considerations as disease entity and prior therapy. The dosage schedule we have followed is shown in TABLE 1. Although many physicians have been able to maintain TEM chemotherapy for months or years without undue risk to the patient, others paying less attention to dosage variation have found it too dangerous to use.

TABLE 1

TEM DOSAGE FOR ORAL ADMINISTRATION*

Schedule:	Day 1	2.5 mg.
	Day 2	2.5 mg.
	Day 3	2.5 mg. *if* preceding doses have not affected WBC or appetite.
	Days 4–7	Withhold TEM, then proceed according to patient's sensitivity.

1. *Extreme sensitivity:* anorexia or fall in WBC after two 2.5 mg. doses. Maintenance, 0.5 to 1.0 mg. every 7 to 14 days.
2. *Average sensitivity:* slight anorexia or fall in WBC after three 2.5 mg. doses. Maintenance, 1.0 to 2.5 mg. every 5 to 10 days.
3. *Low sensitivity:* no effect from first 3 doses. Maintenance, 2.5 or even 5 mg. every 2 to 5 days.

* Maximal single dose, 2.5 to 5.0 mg. Tablets were given to patients fasting, with 2 gm. or more of sodium bicarbonate.

The shortcomings of TEM as a therapeutic agent are less important now that other orally absorbed alkylating agents that are easier and perhaps

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* We are indebted to J. M. Ruegsegger, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y., for generous supplies of TEM.

safer to use, more regular in their dosage effect and, possibly, equally effective have been developed. The chief reason for reviewing the results of TEM therapy of the past six years is to summarize what we have learned about this orally absorbed mustard compound that is important in reference to specific diseases as we study, compare, and evaluate new agents.

There is a fairly general agreement that TEM, along with the other nitrogen mustard compounds, is useful in controlling malignancies that arise from the lymph nodes, bone marrow, and a few other tissues (TABLE 2). Additional or alternative agents commonly employed for local, general, or supplementary purposes are listed in their approximate order of usefulness.

TABLE 2
THERAPEUTIC USE OF TEM

Leukemia, chr. lymphocytic	TEM, steroids, local X ray (P^{32} or spray X ray, steroids)
Lymphoma, giant follicular	
lymphocytic	
lymphoblastic	
lymphosarcoma	
Reticulum cell sarcoma	Local X ray, TEM, steroids
Hodgkin's disease	
Leukemia, chr. granulocytic	TEM or Myleran P32, spray X ray
Polycythmia vera	
Papillary cystadenoca. ovary	TEM, HN2 (I.V. or intrapleural) Thio-TEPA (I.V. or intraperitoneal) Local X ray
Undifferentiated met. carcinoma	

For all purposes chlorambucil may be substituted for TEM.

The therapeutic benefits of TEM have been most impressive generally in chronic lymphocytic leukemia. There is no sharp clinical, pathological, or therapeutic distinction between leukemic and subleukemic forms of lymphocytic leukemia and the lymphocytic and lymphoblastic lymphomas. In the latter, abnormal cellular proliferation tends to be more localized. The results of TEM chemotherapy in the lymphomas are comparable to those in lymphocytic leukemia, although opportunities for effective irradiation therapy are perhaps greater.

In reticulum cell sarcoma and Hodgkin's disease, local irradiation is usually the best primary therapeutic agent. Only a small percentage of patients has responded really well to TEM alone for any length of time. When used in conjunction with local irradiation, however, TEM chemotherapy appears to reduce the frequency of local recurrences and the rate of general dissemination. The mustard compounds TEM and mechlorethamine hydrochloride (HN2) seem to have replaced nearly everywhere the use of whole-body irradiation and/or P^{32} in patients with diffuse or occult disease.

The results of TEM therapy in chronic granulocytic leukemia and polycythemia vera appear to be quite satisfactory in at least 60 to 80 per cent of all patients.[15, 17, 20, 21] The greater convenience and fewer side reactions of Myleran and radioactive phosphorus, however, have made these agents currently more popular. TEM has been of no definite benefit in acute leukemia and in multiple myeloma.

The effects of TEM and other mustard compounds on undifferentiated neoplasms are occasionally worthwhile. The most sensitive tumor arising from extrahematopoietic tissues appears to be ovarian papillary cystadenocarcinoma.[22] About one half of all patients with widespread or metastatic ovarian malignancy are definitely benefited by mustard chemotherapy. Better designed large-scale studies of the effects of mustard compounds and other agents on ovarian, metastatic breast, and undifferentiated bronchus tumors are being undertaken.

During the last two years we have studied the effects of chlorambucil* in over 80 patients with hematopoietic and other malignancies similar to those we have treated with TEM. The newer chemical is easier and perhaps safer to use. We have no evidence, however, that it has a greater therapeutic selectivity or qualitatively different oncolytic properties.

The contribution of TEM to clinical chemotherapy can be illustrated most specifically by its effect in patients with chronic lymphocytic leukemia. In 1949 we attempted to give a consecutive series of patients with this disease nitrogen mustard therapy patterned after Osgood's regularly spaced "titrated" regimen.[23] Side reactions to HN2, the necessity for hospitalization, intravenous therapy, and complicating venous thromboses made it impracticable. When TEM became available in 1950, this regimen could be carried out. The results of maintained TEM therapy and some of the problems encountered in the long-term control of this disease can now be summarized.

The following patient with unusually chronic, almost "benign" lymphocytic leukemia, is typical of about one fourth of all patients with this disease. The abnormal lymphocytic proliferation appears to be controllable by TEM almost indefinitely.

G. W. L., C18920. This 45-year-old Negro farmer developed frequent throat infections and enlarged cervical lymph nodes in 1944. Three years later a node biopsy was interpreted as showing "lymphocytic lymphoma." Blood studies were diagnostic of chronic lymphocytic leukemia. With irradiation therapy the enlarged lymph nodes disappeared and his health became good. In 1951 he again had frequent respiratory infections. He was found to have generalized lymph-node enlargement, splenomegaly, mild anemia, and heavy lymphocytic infiltration of his bone marrow. TEM chemotherapy was started and maintained as shown in FIGURE 1. The patient's physical and hematological abnormalities disappeared. After 6 years of TEM therapy, his health was unimpaired and his hematological status virtually normal.

A second patient with somewhat more severe lymphocytic leukemia is typical of another third or two fifths of individuals with this disease.

G. T., C11185. This 43-year-old female cook had had recurrently enlarged cervical lymph nodes for 2 to 3 years before malaise, weakness, and newly enlarged nodes prompted diagnostic studies that led to the finding of chronic lymphocytic leukemia. When irradiation to the neck appeared to be of little or no benefit to her, she was referred for chemotherapy. After she had

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* Supplied by Donald S. Searle, Burroughs Wellcome & Co., Tuckahoe, N. Y.

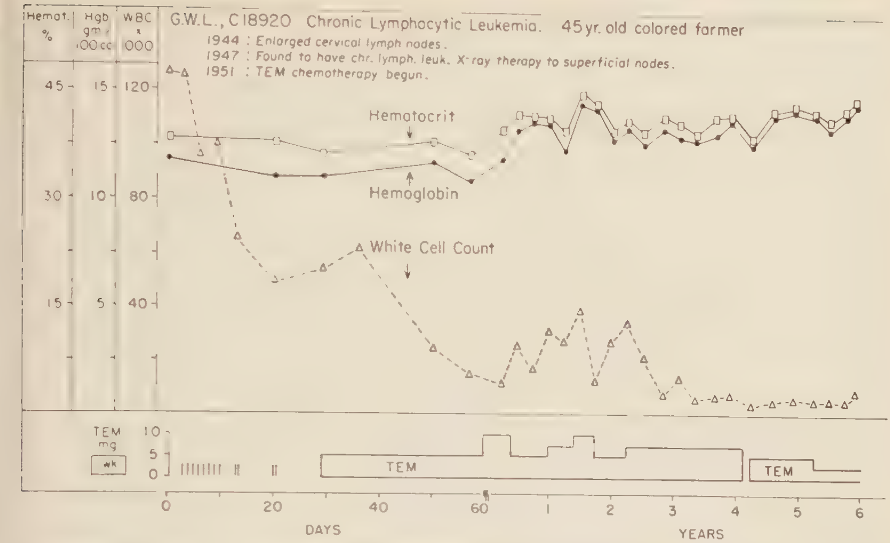

FIGURE 1. Hematological effects of TEM in a patient with chronic lymphocytic leukemia treated over a period of 6 years.

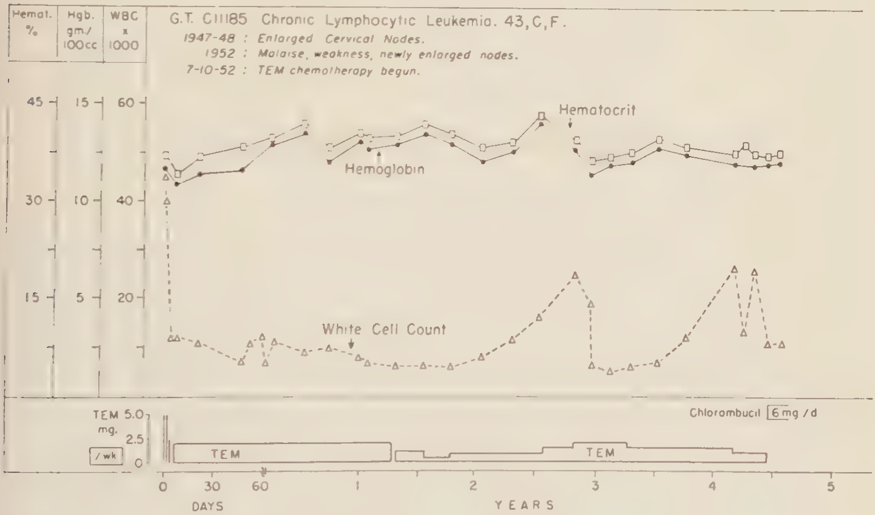

FIGURE 2. Effects of continuous TEM therapy in chronic lymphocytic leukemia maintained for 4½ years, compared with that of chlorambucil.

taken TEM for a few weeks, lymphadenopathy, lymphocytic leukocytosis, mild anemia, and heavy lymphocytic infiltration of the bone marrow all disappeared. After 2½ years of TEM chemotherapy her disease became more active and difficult to control (FIGURE 2). At 4½ years, chlorambucil was substituted for TEM. The therapeutic effects of the two agents appeared to be equivalent when compared over a period of several months.

In contrast to their high frequency in chronic granulocytic leukemia, acute exacerbations of chronic lymphocytic leukemia are comparatively rare. They may be seen more frequently in the future if early complications are averted and the duration of the disease is prolonged.

N. W. S., D74896. This 58-year-old housewife was found to have an abnormal lymphocytic leukocytosis after being admitted to the hospital for a hemorrhoidectomy on November 10, 1953. The lymphocytes in her circulating blood had prominent nucleoli and were unusually immature for chronic leukemia. With TEM therapy her disease was fairly well controlled for two years (FIGURE 3). This was followed by an acute exacerbation of her disease that terminated fatally in spite of energetic steroid, 6-mercaptopurine, and transfusion therapy.

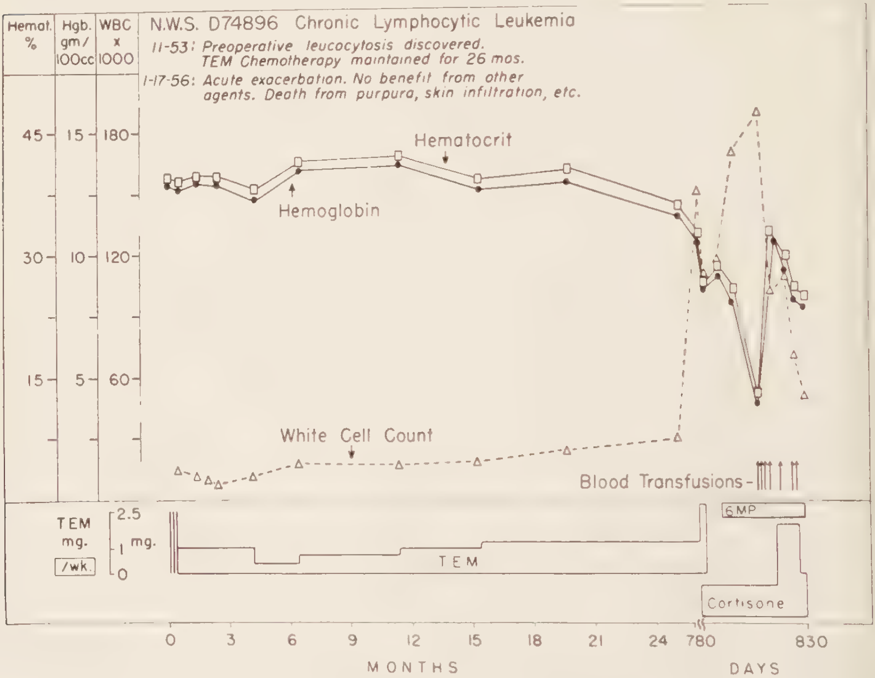

FIGURE 3. Acute fatal exacerbation of chronic lymphocytic leukemia occurring after 2 years of TEM therapy.

About one patient in five with chronic lymphocytic leukemia requires steroid therapy in addition to an oral mustard compound for satisfactory control of their disease. Benefit from steroids occurs even in patients with a negative Coombs test and no obvious evidence of accelerated red cell hemolysis.

J. T. S., E15121. This 74-year-old farmer, while being treated for a fracture of the mandible, was found to have leukocytosis with abnormal lymphocytes in the circulating blood. For one to two years his health had been deteriorating. On examination there were no enlarged lymph nodes, but the spleen was palpable. He was mildly anemic and his marrow was heavily infiltrated with lymphocytes. For a period of six months he responded well to TEM therapy, but then began to lose weight and become more anemic. The Coombs test remained negative and there was no obvious evidence of accelerated red cell hemolysis. The TEM dose was increased and prednisolone was added to his regimen (FIGURE 4). He improved quickly, but relapsed after the steroid was withdrawn. Chlorambucil and prednisolone* were then used concurrently. During the following year his physical and hematological status remained excellent.

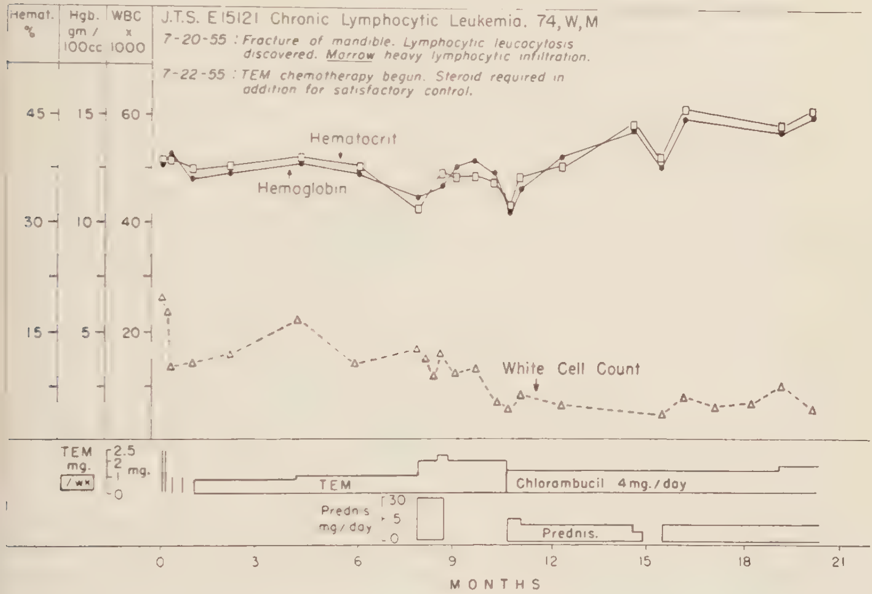

FIGURE 4. Chronic lymphocytic leukemia requiring steroid therapy in addition to an oral mustard compound.

O. S. J., B71682. This 58-year-old farmer first developed enlarged cervical lymph nodes in 1949. Biopsy led to the diagnosis of lymphosarcoma and, with local X-ray therapy, the nodes disappeared. Three years later he became disabled with signs of congestive heart failure. He responded poorly to a variety of agents used over short periods of time at home. On examination he had massive enlargement of the lymph nodes and spleen. His hemoglobin was reduced to 8.4 gm. per cent and the hematocrit to 26.8 per cent. The WBC was 9700, with 73 per cent abnormal lymphocytes. In the bone marrow, normal elements were greatly reduced in number, and there was heavy lymphocytic infiltration. The Coombs test was negative. With TEM and cortisone therapy, his clinical and hematological status improved (FIGURE 5). He relapsed, however, after TEM was suspended, despite the continued use of cortisone, and required a series of blood transfusions. With the concurrent use of chlorambucil and prednisolone, he improved again and remained well during the subsequent 9 months.

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* Supplied as Sterane by M. W. Amster, Pfizer Laboratories, Chas. Pfizer & Co., Inc., Brooklyn, N. Y.

An ominous complication in chronic lymphocytic leukemia is infiltration and replacement of the bone marrow with abnormal lymphocytes, which predisposes to anemia, thrombocytopenia, and/or neutropenia. This may occur as a late manifestation of uncontrolled disease or may represent a complication that occurs despite maximal therapy in patients with aggressive disease. Some good therapeutic results have been seen for the first time in recent years following the administration of steroids and the cautious use of TEM or chlorambucil. Only a few patients with severe leukemic marrow damage, however, when given both steroids and TEM therapy, recover to the degree illustrated by the following patient:

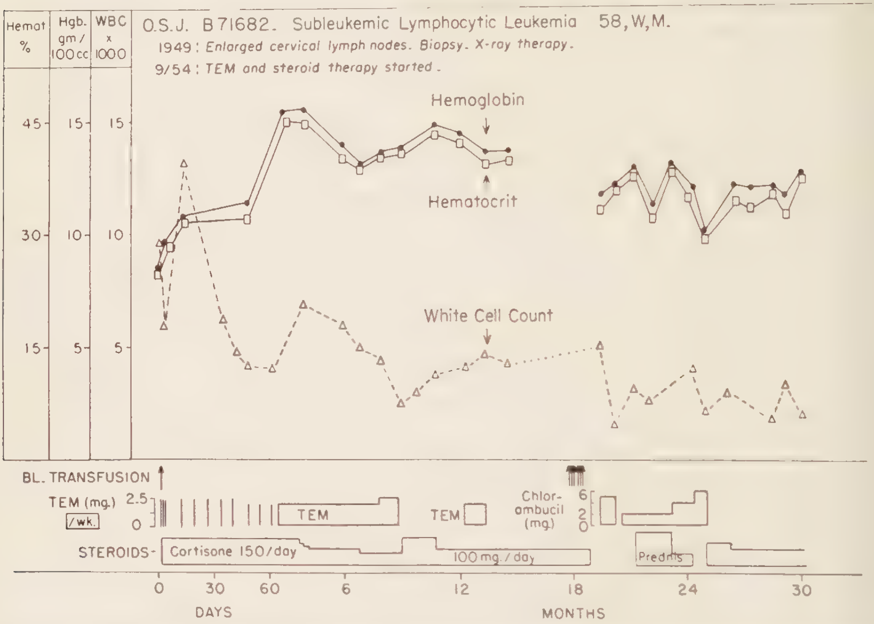

FIGURE 5. Hematological response of a patient with subleukemic lymphocytic leukemia to cortisone and TEM. A relapse on cortisone alone was followed by good control with chlorambucil and prednisolone.

L. W., D51066. This 57-year-old housewife developed symptoms of anemia in May 1952, and her condition deteriorated steadily during the following months. When seen in November 1952 she was found to have slight enlargement of the superficial lymph nodes, splenomegaly, and reduction of the hemoglobin concentration to 2.7 gm. per cent and of the hematocrit to 8.3 per cent. Her WBC was 135,000. Bone marrow aspirated from the sternum contained virtually nothing but lymphocytes. She was given blood transfusions and TEM in amounts sufficient to reduce the WBC to normal (FIGURE 6). Within a month, however, anemia recurred, and additional blood transfusions were required. Cortisone was then added to her regimen. The hemoglobin, red cell count, and hematocrit were maintained at satisfactory levels spontaneously for a period of 9 months, but fell when steroid therapy was suspended. Despite the resumption of cortisone, a series of infections and anemia led to her death 18 months after her first visit.

The total number of our patients with chronic lymphocytic leukemia treated with TEM and the estimated effectiveness of the agent in controlling their abnormal lymphocytic proliferation are summarized in TABLE 3. One hundred and twelve patients were treated from 1950 through 1956. The 89 "leukemic" individuals had total leukocyte counts of 15,000 or more before therapy was started. Of this group, 27 per cent had excellent or virtually complete remissions with TEM. Forty-three per cent had good remissions, although minor lymph node, splenic enlargement, anemia and/or lymphocytosis persisted. In an additional 16 per cent TEM was of some benefit although, by itself, it was inadequate to control the disease satisfactorily. Patients with subleukemic leukemia appeared to respond less favorably than those in whom the peripheral white blood counts were higher.

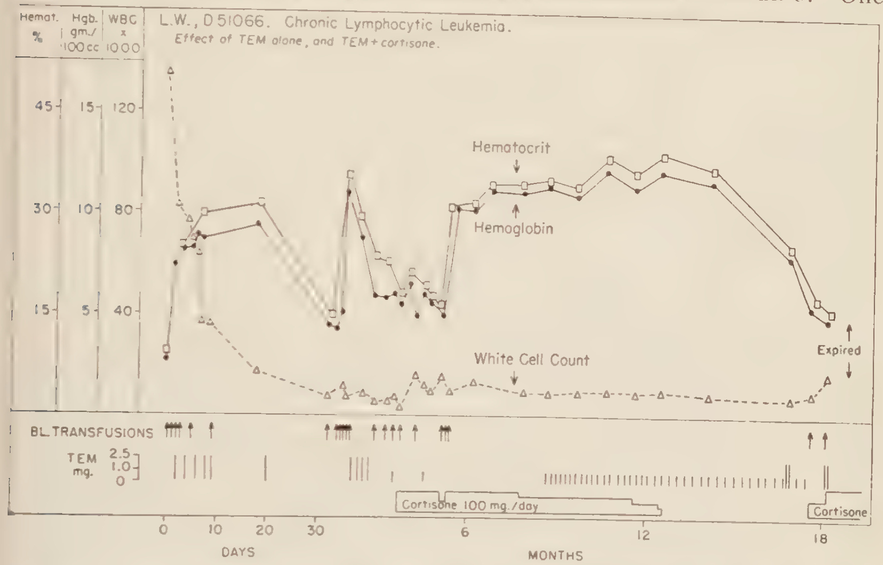

FIGURE 6. Hematological effects of TEM and TEM plus cortisone in a patient with lymphocytic leukemic marrow replacement.

The effect of TEM on the bone marrow of patients with chronic lymphocytic leukemia is especially important since (1) adverse effects impose strict limits on the amount of chemotherapy that can be given and (2) the elimination of lymphocytic infiltration without depression of bone marrow function is the best index of antileukemic selectivity.

Bone marrow aspirations were carried out in 109 of our patients before treatment and, in all but 5 instances, abnormal lymphocytic infiltration was

TABLE 3

PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA (DUKE HOSPITAL, 1950–1956) TREATED WITH TEM

Total number of patients	112
Leukemic group, WBC over 15,000	89
Subleukemic group, WBC under 15,000	23

Effectiveness of TEM in control of disease

	Leukemic group	Subleukemic
Excellent	24 (27%)	1
Good	38 (43%)	7
Fair	14 (16%)	9
Questionable	9	4
No effect	4	2
Steroids needed	12	5
Coombs test positive	4	0

present. Marrow examinations repeated during TEM administration in 68 patients showed disappearance of lymphocytic foci in 11, significant reduction in the number of lymphocytes in 29, no change in 8, and progressive infiltration and replacement in 4. Normal myeloid and erythroid elements were virtually replaced by lymphocytes in 16 patients. Despite control of the peripheral lymphocytosis in these individuals, only 3 had any significant regeneration of bone marrow.

Sixty-eight patients in this series were not anemic when therapy was begun. Ten became anemic within 1 year, due to inadequate control of their disease. Twenty-eight patients had moderately severe anemia at the beginning of treatment. During the course of chemotherapy, 11 of these gained 2 gm. per cent or more in hemoglobin concentration and 5 per cent or more in packed red-cell volume. Anemia persisted without material change in the remaining 17. Only 3 of the 16 patients with leukemic marrow replacement had significant improvement in anemia, thrombocytopenia, and/or neutropenia after treatment.

Convincing data regarding the effect of therapy on the survival of patients with chronic lymphocytic leukemia has been difficult to obtain. For ou TEM series, as with others, there is no available group of concurrent controls treated differently and/or untreated. Uniform handling of a consecutive series of patients does minimize selection factors, but the random choic of agents to be studied concurrently is obviously most desirable. If, however, one were to compare the effect of one mustard compound with anothe or with an irradiation therapy regimen, many present-day patients woul have to be given steroids eventually. The analysis of therapeutic accomplishments over a period of years will continue to pose many difficulties.

Some inference regarding the effect of TEM on the survival of patient with chronic lymphocytic leukemia may be provided by the patients whor

TABLE 4

SURVIVAL OF PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA TREATED WITH TEM, 1950–1953

Total number of patients	54
75% survival at	28.5 mo.
50% survival at	54 mo.
33% survival, March 1957, average	74.4 mo.
TEM given only during last 1 to 6 mo. to	10 patients
Death due to causes other than leukemia	8 patients
Death from previous marrow damage	9 patients

we started to treat during the years 1950 through 1953 (TABLE 4). While most of these 54 patients were given only TEM, a few had some local irradiation and or steroid therapy. Seventy-five per cent of the group were living 28.5 months after the onset of their disease, and 50 per cent at 54 months. One third were still living in March 1957, with an average survival of 74.4 months. It should be noted that our series included 10 patients treated for only a comparatively short period of time before death, and 8 who died of causes other than leukemia. Nine patients died from bone marrow damage present before the start of chemotherapy.

The statistics compiled by Shimkin and his collaborators[24] on the survival of patients with chronic lymphocytic leukemia show that no significant improvement resulted from conventional therapy from 1913 to 1947. With the early, cautious, and persistent use of whole-body or P^{32} irradiation, Osgood *et al.*[23, 25] and Lawrence[26] have reported considerable improvement in longevity. Our present indications regarding the comparative efficacy of the oral mustard compounds are to the effect that when these are used in a comparable manner the over-all results are at least as good.

Summary

Triethylene melamine, the first nitrogen mustard-like compound found to be suitable for oral administration, represents an important advance in practical cancer chemotherapy.

TEM has been a most effective agent in the treatment of chronic lymphocytic leukemia, and a valuable supplement to local irradiation in malignant lymphomas and Hodgkin's disease. In chronic granulocytic leukemia and polycythemia vera, good therapeutic results have been obtained in the majority of cases. About 50 per cent of the patients with widespread ovarian carcinomas and a few with undifferentiated metastatic carcinomas have been benefited significantly by TEM chemotherapy.

A major deterrent to the general clinical use of TEM has been variable dosage effects in different patients. When compared with chlorambucil, the latter compound is easier and perhaps safer to use. No great difference in antitumor selectivity of the two compounds has been observed.

References

1. LEWIS, M. R. & M. L. CROSSLEY. 1950. Retardation of tumor growth in mice by oral administration of ethylenimine derivatives. Arch. Biochem. **26:** 319.

2. Burchenal, J. H., M. L. Crossley, C. C. Stock & C. P. Rhoads. 1950. The action of certain ethylenimine (aziridine) derivatives on mouse leukemia. Arch. Biochem. **26**: 321.
3. Rose, F. L., J. A. Henry & A. L. Walpole. 1950. New cytotoxic agents with tumor-inhibiting activity. Nature. **165**: 993.
4. Philips, F. S. & J. B. Thiersch. 1950. The nitrogen mustard-like actions of certain ethylenimines. I. 2,4,6-Tris(ethylenimine)-s-triazine and analogues. J. Pharmacol. Exptl. Med. **100**: 398.
5. Karnofsky, D. A., J. H. Burchenal, G. C. Armistead, Jr., C. M. Southam, J. L. Bernstein, L. F. Craver & C. P. Rhoads. 1951. Triethylene melamine in the treatment of neoplastic disease. A.M.A. Arch. Internal Med. **87**: 477.
6. Paterson, E. & J. Boland. 1951. Trisethyleneimino-s-triazine in human malignant disease: preliminary trial. Brit. J. Cancer. **5**: 28.
7. Bayrd, E. D., J. M. Stickney, B. E. Hall & C. H. Watkins. 1952. Clinical observations on the use of triethylene melamine. Cancer. **5**: 336.
8. Conference on Triethylene Melamine. 1952. Acta Haematol. **8**: 116.
9. Gellhorn, A., M. M. Kligerman & I. Jaffe. 1952. Triethylene melamine in clinical cancer chemotherapy. Am. J. Med. **13**: 428.
10. Kravitz, S. C., H. D. Diamond & L. F. Craver. 1952. The clinical use of triethylene melamine. Blood. **7**: 729.
11. Rundles, R. W. & W. B. Barton. 1952. Triethylene melamine in the treatment of neoplastic disease. Blood. **7**: 483.
12. Silverberg, J. H. & W. Dameshek. 1952. Use of triethylene melamine in treatment of leukemia and leukosarcoma. J. Am. Med. Assoc. **148**: 1015.
13. Wright, J. C., A. Prigot, L. T. Wright & I. Arons. 1952. A.M.A. Arch. Internal Med. **89**: 387.
14. Axelrod, A. R., L. Berman & R. V. Murphy. 1953. Clinical and hematologic effects of triethylene melamine. Am. J. Med. **15**: 684.
15. Bond, W. H., R. J. Rohn, R. W. Dyke & P. J. Fouts. 1953. Clinical use of triethylene melamine. A.M.A. Arch. Internal Med. **91**: 602.
16. Burtner, O. W. 1953. Triethylene melamine in the treatment of lymphomas and other neoplastic diseases. Ann. Internal Med. **38**: 1222.
17. Karnofsky, D. A. 1954. Triethylene melamine in the treatment of lymphomas and leukemia. Med. Clin. N. Am. : 541.
18. Walsh, J. R., P. T. Pratt, W. E. Graham & H. J. Zimmerman. 1954. Treatment of leukemia and lymphomata with triethylene melamine. Acta Haematol. **11**: 329.
19. Hambly, C. K. & T. I. Robertson. 1955. Triethylene melamine (TEM) in the treatment of malignant lymphoid disease. Med. J. Australia. **1**: 900.
20. Rosenthal, N. & R. L. Rosenthal. 1952. Treatment of polycythemia vera with triethylene melamine; summary of thirty cases. A.M.A. Arch. Internal Med. **90**: 379.
21. Ellison, R. R., V. Ginsberg & J. Watson. 1953. Triethylene melamine in polycythemia vera. Cancer. **6**: 327.
22. Sykes, M. P., R. W. Rundles, V. K. Pierce & D. A. Karnofsky. 1955. Triethylene melamine in the management of far advanced ovarian cancer. Surg. Gynecol. Obstet. **101**: 133.
23. Osgood, E. E. & A. J. Seamon. 1952. Treatment of chronic leukemias. J. Am. Med. Assoc. **150**: 1372.
24. Shimkin, M. B., E. L. Lucia, K. C. Oppermann & S. R. Mettier. 1953. Lymphocytic leukemia: an analysis of frequency, distribution and mortality at the University of California Hospital, 1913–1947. Ann. Internal Med. **39**: 1254.
25. Osgood, E. E., A. J. Seamon & R. D. Koler. 1956. Natural history and course of the leukemias. Proc. 3rd Natl. Cancer Conf. : 366.
26. Lawrence, J. H. 1954. The treatment of chronic leukemia. Med. Clin. N. Am. : 525.

SUMMARY OF RESULTS WITH TRIETHYLENE THIOPHOSPHORAMIDE*

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Triethylene thiophosphoramide† (*N,N′,N″*-triethylene thiophosphoramide (TSPA, Thio-TEPA) is the sulfur derivative of triethylene phosphoramide. Like triethylene melamine (TEM) and triethylene phosphoramide (TEPA), it is a member of the class of compounds known as the ethylenimines. The phosphoramide derivatives were synthesized by the American Cyanamid Research Laboratories, Pearl River, N. Y.[1] FIGURE 1 shows the similar chemical configurations of TEM, TEPA, and TSPA.

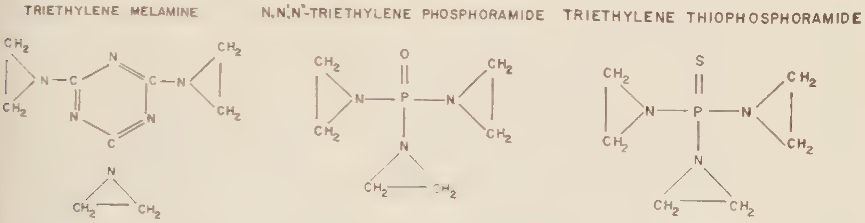
TRIETHYLENE MELAMINE
N,N',N''-TRIETHYLENE PHOSPHORAMIDE
TRIETHYLENE THIOPHOSPHORAMIDE

FIGURE 1

The antitumor activity of the phosphoramides was demonstrated by Buckley *et al.*[2] against mouse sarcoma 180, by Crossley *et al.*[3] against rat sarcoma, and by Burchenal *et al.*[4] in prolonging the survival time of leukemic mice. Personeus and his associates[5] found Thio-TEPA to be the most effective in inhibiting metastases of rat mammary adenocarcinomas. Activity by Thio-TEPA was found against chloroleukemia in rats by Crossley *et al.*,[6] and against the Flexner-Jobling carcinoma in Sprague-Dawley rats by Sparks *et al.*[7] Subsequently a wide variety of mouse and rat tumors were observed to be inhibited by this compound.[8-11]

Results of the use of TEPA in clinical cancer chemotherapy were first reported by Farber *et al.*[12] and Sykes *et al.*[13] These and other investigations[14-15] indicated important degrees of improvement in patients with Hodgkin's disease, lymphosarcoma, the chronic leukemias, neuroblastoma, and malignant melanomas treated with TEPA. TEPA and other early phosphoramides, namely diethylene phosphoramide (DEPA) and Oxa-DEPA, which produced results similar to those of Thio-TEPA, were replaced by Thio-TEPA because of its greater stability.[1]

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In 1953, Shay and his associates[16] first reported on the clinical effectiveness of Thio-TEPA. The best results in their studies[16-20] were observed in patients with chronic myelogenous leukemia, chronic lymphatic leukemia, and carcinoma of the breast and ovary. Lesser improvement occurred in subacute leukemia, Hodgkin's disease, lymphosarcoma, multiple myeloma, carcinoma of the stomach, and malignant melanoma. In one of these studies[17] of 47 cases of carcinomas, 6 out of 12 with breast carcinoma, 2 out of 3 with ovarian carcinoma, and 2 with malignant melanoma responded.

A series of reports by Bateman *et al.*[21-26] indicate striking palliation in patients with far-advanced mammary carcinoma and carcinoma of the ovary treated with Thio-TEPA. In one of these series, which totals 94 patients,[23] good results were achieved in 25 of 32 mammary carcinomas, 6 of 12 ovarian carcinomas, 1 of 4 cervix carcinomas, and 3 of 5 glioblastomas. In another series of 99 cases[21] objective improvement was reported in 4 of 10 cases with lung carcinoma, but little or no benefit in cases with gastrointestinal, genitourinary, or head and neck tumors. Clinical improvement was observed in 80 per cent of this group of patients. The improvement included "increase in sense of well-being, appetite and weight gain; decrease in pain, dyspnea, dysphagia and cough; control of pleural effusion and ascites; regression of tumor masses and regional adenopathy; recalcification of bone lesions and control of neurologic signs and symptoms."[21] Pleural and ascitic fluid was controlled with Thio-TEPA in patients with breast and ovarian carcinoma.[24] In 17 of 22 patients with different types of ovarian carcinoma[26] varying degrees of improvement from control of ascites to disappearance of ascites and masses occurred. The average duration of improvement in this group was 8 months.

Sykes and her associates,[27] in a series of 30 of 46 cases treated sufficiently for evaluation with Thio-TEPA, obtained subjective and objective improvement in 6 cases (2 of these were breast carcinomas, 1 ovarian, 1 bronchogenic, 1 reticulum cell sarcoma, and 1 chronic lymphatic leukemia).

The experience of Farber *et al.*[1] with Thio-TEPA shows the compound to have important temporary effects on malignant melanoma and on seminoma metastases resistant to irradiation.

Unpublished data of Ultmann and his co-workers[28] on the results with Thio-TEPA on 100 patients indicate objective improvement in 13 of 27 ovarian carcinomas, 19 of 43 malignant lymphomas, and no improvement in 9 patients with carcinoma of the breast, 3 with carcinoma of the liver, 3 with carcinoma of the lung, and 5 with malignant melanoma.

This report constitutes our observations on the use of Thio-TEPA ir 94 patients with a variety of incurable, histologically proved, neoplastic diseases. Some of these cases have been reported previously.[29, 30]

Preparation of Thio-TEPA

Triethylene thiophosphoramide is a dry white crystalline compound solubl in water at room temperatures. For clinical use it is dissolved in sterile dis tilled H_2O or in physiological saline to make a concentration of 10 mg. per ml The solution is then sterilized by passing it through a sintered glass filter o

UF porosity and is tested by routine bacteriological methods. It is stored in sterile, rubber-capped vials at 2° C. for a maximum period of 6 weeks.

Clinical Dosage of Thio-TEPA

Various methods of administration and dosage schedules have been used and reported. In general, the patients in our series of 94 cases received doses of Thio-TEPA varying between 5 and 40 mg., with an average of 10 to 20 mg., at weekly intervals, providing the white blood count was 5000 or more. The drug was administered intramuscularly to 82 patients, into the tumor to 25 patients (of these 19 also received the drug intramuscularly), intraperitoneally to 6 patients (of these 4 also received the drug intramuscularly), orally to 6 (all of these also received the drug intramuscularly), intrapleurally to 3 (2 also received the drug intramuscularly), and intrathecally to 1. Therapy was continued for months, with discontinuance when the level of the white blood count fell below the 5000 level. When the white blood count was below 5000, therapy was not resumed until the count returned to normal, when it was resumed at lower weekly dosages than previously. The sensitivity of the hematopoietic systems of different patients to Thio-TEPA, like the sensitivity to TEM and TEPA, varied greatly. Dosage schedules were adjusted frequently for each patient according to his or her sensitivity to the drugs. When patients were in good general condition at the onset of therapy, the initial intramuscular or intratumor dose was 40 mg. Patients who had received prior irradiation or who were immediately postoperative were given a small initial dose of 10 mg. Patients to whom prior irradiation had been given tolerated smaller doses of Thio-TEPA. Recent postoperative patients were given small initial doses to prevent leukopenia and its complications, for example, infections, during the recovery period.

TABLE 1
DOSE OF THIO-TEPA FOR IMPROVEMENT

Dose (mg.)	Number of cases
25–49	1
50–74	4
75–99	3
100–124	8
125–149	4
150–174	3
175–199	7
200–224	1
225–249	2
250–274	1
275–299	2
300–324	0
325–349	2
350–374	0
More than 375	1

When improvement occurred following the administration of Thio-TEPA, it was generally observed between the seventh and thirtieth day succeeding initiation of therapy. In 2 patients, improvement was not observed until the forty-second day; in 1 patient it took 95 days. In 1 patient with Hodgkin's disease, it required 90 days to achieve a complete remission. This delay in improvement was similar to our experience with TEM and TEPA, where improvement occurred between the third and the twenty-eighth day, but complete remissions required between 3 to 9 months.[30]

The total dose of Thio-TEPA administered in our series of patients varied from 10 to 780 mg. Of these, 16 cases received 10 to 49 mg., 18 from 50 to 99 mg., 31 from 100 to 199 mg., 14 from 200 to 299 mg., 7 from 300 to 399 mg., 2 from 400 to 499 mg. and 4 received doses over 500 mg. Table 1 shows the dosages employed in this series to produce and maintain improvement. Forty mg. was the smallest dose, and 555 mg. the largest. The duration of therapy ranged from 1 to 318 days. Five cases treated are still alive after more than one year, and one case has survived more than 2 years since the institution of therapy.

Toxicity

Thus far, the only observed side effect of Thio-TEPA therapy in our series of cases has been the development of hematological depression. Usually this has consisted of only a moderate depression in the level of the total white blood count. When the total white blood count falls to low levels the platelets are also likely to decrease. It is known that, if Thio-TEPA dosage schedules are not adjusted properly for each patient, severe hematological depression with its clinical sequelae will occur. In our series of 94 cases treated with Thio-TEPA, 59 developed white blood count levels below 5000 one or more times. The remaining 35 patients did not develop leukopenia. The white blood count levels fell to between 4000 and 4999 in 20 instances, 3000 to 3999 in 32 instances, 2000 to 2999 in 19 instances, and 1000 to 1999 in 9. No patient in our series developed a leukopenia of under 1000.

Table 2 shows the individual white blood count level, dose, and duration of Thio-TEPA therapy that produced leukopenia. Table 3 shows the total dose administered prior to the development of leukopenia and the number of instances in which leukopenia occurred. The smallest total dose that produced this condition was 10 mg., the largest was 570 mg. Table 4 shows the number of days from the institution of therapy to the time when leukopenia developed, and the number of instances in which leukopenia occurred. In 8 cases it occurred in less than 10 days, and in 3 it occurred in 99 to 155 days.

Table 5 shows the time in weeks from leukopenia until the white blood count returned to 5000 or over. In the majority of cases the white blood count returned to normal in 1 to 2 weeks; in 3 cases, however, it required 7 weeks. All cases in which leukopenia developed recovered except for 5 with white blood counts ranging from 1000 to 3450. These patients were in terminal condition and died, apparently of their neoplastic disease, evidencing no hemorrhages, purpura, or infection. Of the 5, one had a platelet

TABLE 2

PATIENTS SHOWING LEUKOPENIA WITH TRIETHYLENE THIOPHOSPHORAMIDE

Case	C.R. No.	Dose (mg.)	Days	WBC produced	Days for WBC to return to 5000 or over
3	150	90 I.M. or into tumor	23	3500	7
5	445	25 I.M.	25	2950	Expired same day
8	402	160 I.M.	49	3950	19
		95 into tumor	43	2750	7
9	60	50 I.M.	16	1350	21
10	2	240 I.M.	48	3700	15
11	165	70 I.M.	28	1450	13
12	152	30 I.M.	21	4850	5
13	15	60 I.M.	18	3650	100*
		40 I.M.	10	2550	10
		40 I.M.	13	2700	9
14	5	20 I.M.	21	4700	7
		55 I.M.	35	4150	7
15	22	80 I.M.	38	3900	43
21	148	100 I.M.	34	3300	23
22	437	100 I.M.	21	1050	10
23	448	110 I.M. & intrapleur.	25	3600	14
27	433	120 I.M.	28	1150	Expired on this day
28	387	180 I.M.	47	2950	11
29	82	100 into tumor	25	3300	10
		60 into tumor	24	2700	In 4 days to 4350. Patient transferred
30	401	90 I.M.	46	3100	4
31	407	80 I.M.	30	2050	19
32	441	90 I.M.	15	4650	3
		30 I.M.	1	3650	2
34	426	70 I.M.	15	3950	7
36	120	80 I.M.	29	2150	In 7 days to 4100 (under study)
37	153	50 I.M.	20	4550	7
40	21	70 I.M.	43	3800	14
		55 I.M.	80	3050	7
41	437	110 I.M.	24	2750	43
		80 into tumor	22	1600	26
42	414	570 I.M. & intraperi.	112	4400	4
43	57	170 I.M.	81	1900	14 to 4550
44	34	160 I.M.	34	3200	7 to 4700
45	11	30 I.M.	12	3850	3 to 4950
		10 intraperi.	22	2700	21
46	75	80 I.M.	19	1000	Expired same day
48	128	90 intrapleur.	36	3000	7
		30 intrapleur.	3	3000	Expired. WBC increased to 3100
49	140	120 I.M.	26	2800	16
50	443	45 I.M.	20	4050	15
53	444	140 I.M.	38	1900	Expired—2 days
54	399	180 I.M.	60	3050	6
56	400	10 I.M.	7	3950	3
59	376	70 I.M.	28	3850	26

* Patient failed to return for check-up sooner.

TABLE 2 (*Concluded*)

Case	C.R. No.	Dose (mg.)	Days	WBC produced	Days for WBC to return to 5000 or over
60	422	10 I.M.	20	2200	Expired same day
62	439	80 I.M.	21	3100	14
		30 I.M.	11	3150	12
64	8	50 I.M.	14	4600	5
		30 into tumor	4	4850	12
		60 into tumor	16	4000	7
65	442	20 I.M.	10	3500	14
		20 into tumor	6	2650	22
		20 into tumor	16	2300	4
66	355	30 I.M.	27	2900	14
67	56	235 I.M. & into tumor once	117	2000	27
68	31	195 into tumor & I.M.	49	3650	14
		175 into tumor & I.M.	63	2500	7
69	106	370 into tumor	81	4600	7
70	135	180 into tumor & I.M.	49	3150	20
71	366	30 I.M.	7	3450	Expired at this time
72	38	145 into tumor & I.M.	22	4150	7 to 4900
		20 I.M.	11	3150	11
73	42	160 I.M.	25	1400	7
74	47	150 I.M.	36	4650	7
76	52	15 into tumor	16	4100	7
		45 into tumor & I.M.	25	3350	17
		10 I.M.	28	3750	6
		120 I.M.	56	4400	14
		55 into tumor	37	2750	29
77	67	150 I.M.	34	4000	Expired on this day
78	71	80 into tumor & I.M.	41	2900	45
79	74	110 I.M.	21	4600	7
80	104	100 into tumor & I.M.	13	4450	8
87	81	120 intrathecally	38	3000	25
88	432	120 intraperi.	14	4100	2
89	436	60 I.M.	26	2400	24
91	30	180 I.M.	48	3050	14
93	371	90 I.M.	27	4250	5
		145 I.M.	155	3250	9

count of 60,000, one of 80,000, and the others of more than 110,000. Si patients in the series recovered from counts ranging from 1000 to 2000. It i our impression that Thio-TEPA can be given safely over a long period of tim if the patient's hematopoietic system and general status are frequentl observed and if the dosage is properly adjusted.

Results of Thio-TEPA

In this study, pretreatment and follow-up examinations consisted of fre quent observation of the patient for changes, with particular emphasis upo any changes in size, visibility, or palpability of tumor masses. Repea

TABLE 3
THIO-TEPA TOXICITY

Dose (mg.)	Number of cases that developed leukopenia
0–24	10
25–29	13
50–74	14
75–99	13
100–124	12
125–149	3
150–174	6
175–200	5
200 & more	4

TABLE 4
THIO-TEPA TOXICITY DATA

Days from onset of therapy	Number of cases that developed leukopenia
0–9	8
10–19	18
20–29	26
30–39	10
40–49	9
50–59	2
60–69	1
70–79	1
80–89	2
90–155	3

TABLE 5
RECOVERY TIME FROM THIO-TEPA LEUKOPENIA

Number of weeks until WBC returned to normal	Number of cases
1	33
2	17
3	9
4	7
5	1
6	0
7	3
8 & more	1*

* Patient failed to return for follow-up sooner.

biopsies, complete blood counts, complete blood chemistries, X rays, and other procedures were accomplished as frequently as was considered necessary. Except in a few instances, radiation and other therapy were not given with chemotherapy. Antibiotics were given for intercurrent infection. Transfusions, infusions, narcotics, and vitamins were given when necessary as part of the total care of the patients. Every effort was made to maintain the nutritional status and the comfort and general well-being of each patient in this study.

TABLE 6

RESULTS OF TREATMENT WITH TRIETHYLENE THIOPHOSPHORAMIDE

Diagnosis	Total treated	Regressions in tumor size*	Questionable improvement or *status quo*	Downhill
Rhabdomyosarcoma	2	0	0	2
Kaposi's sarcoma	1	0	1	0
Osteogenic sarcoma	1	1	0	0
Multiple myeloma	1	0	0	1
Chronic myelogenous leukemia	2	2	0	0
Lymphosarcoma	4	1	0	3
Hodgkin's disease	5	3	1	1
Chronic lymphatic leukemia	2	2	0	0
Ca. ear	1	0	0	1
Ca. lung	4	0	2	2
Ca. oropharynx	6	2	0	4
Ca. stomach	3	1	0	2
Ca. large bowel & rectum	9	0	3	6
Ca. pancreas	1	0	0	1
Ca. gall bladder & kidney	1	1	0	0
Ca. testes	1	0	0	1
Ca. ovary	5	2	1	2
Ca. uterus	2	0	1	1
Ca. cervix	2	0	0	2
Ca. breast	17	5	1	11
Malignant melanoma	16	5	1	10
Undiagnosed tumor of spinal cord	1	1	0	0
Metastatic ca. (primary site?)	7	1	1	5
Total	94	27	12	55

* Includes cases showing modest to marked objective evidence of tumor regression in one or more areas.

The criteria used to evaluate the results of Thio-TEPA in this series were based on objective evidences of tumor regression in one or more areas of the body without regard to subjective changes. Patients showing either marked or modest regressions were grouped together (TABLE 6). Included in this group were also patients in whom one or more tumor nodules regressed without progression of the disease in other areas of the body. Patients whose tumors were questionably smaller or whose condition remained unchanged during therapy were considered as a separate group. Patients in whom lesions regressed in size in one area of the body and increased in size in another area of the body were considered unimproved. Our patients were assessed in this way because they presented such a therapeutic challenge that we believed even slight improvement or maintenance of *status quo* was worth reporting as such. It is understood that in some cases human tumors do appear to remain stationary without the use of chemotherapeutic agents.

In this series of 94 cases, 15 had received radiation therapy that ended 6 months or less prior to chemotherapy. Of these, 7 did not respond to Thio-TEPA therapy. The remaining 8 who did respond had (1) lymphosarcoma radiation therapy ended 4 months previously, but the tumor had exacerbated; (2) carcinoma of the ovary -700 r (a small dose) 8 days prior to therapy; (3) 2 cases of carcinoma of the breast radiation therapy ended 6 months previously, but the tumors had subsequently exacerbated; (4) spinal cord tumor (medulloblastoma ?) -radiation therapy ended 2 months previously, but exacerbation had occurred; (5) malignant melanoma -radiation therapy ended 2 weeks before; and (6) carcinoma of the breast therapy ended 5 weeks before. These last 3 cases demonstrated only questionable improvement or remained unchanged on Thio-TEPA therapy.

Six patients to whom chemotherapy or hormones had been given prior to Thio-TEPA therapy showed improvement or remained stationary on Thio-TEPA therapy.

In an attempt to return the blood count to normal when leukopenia occurred, 4 patients received Meticorten* (prednisone). Three of these 4 patients, in whom Thio-TEPA responses had been noted, had shown their improvement prior to Meticorten therapy.

One patient to whom diethylstilbestrol had been given for 7 months prior to Thio-TEPA therapy was becoming worse during this period. For several weeks at the start of Thio-TEPA therapy she improved while the estrogen was also being given. The improvement was maintained after the estrogen was stopped but while the Thio-TEPA was still being administered.

Of the 94 cases in this series treated with Thio-TEPA, objective tumor regression was observed in 27, questionable improvement or maintenance of a stationary condition in 12, and a downhill course with progression of tumors in 55. Table 6 shows the results of treatment with Thio-TEPA and the types of cases included in the study.

In the 27 patients who displayed objective tumor regression, only 1 patient, with Hodgkin's disease, experienced a complete remission. One patient with an ovarian carcinoma showed complete regression of all demonstrable disease. One patient with proved recurrent malignant melanoma with 2 subcutaneous nodules on the leg received injections directly into the tumor and the nodules disappeared. In the remaining 24 cases the improvement was only moderate.

The tumor regressions observed in 5 of the total of 17 cases of breast carcinoma were modest and transitory. It is interesting to note than in 4 of these cases the soft-tissue tumor regression occurred following intratumoral injections of the drug. One of these patients had been treated initially with intramuscular injections without response.

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* Schering Corporation, Bloomfield, N. J.

TABLE 7

ANALYSIS OF 39 CASES SHOWING OBJECTIVE IMPROVEMENT, QUESTIONABLE IMPROVEMENT, OR *Status Quo*

Case No. C.R. No. Diagnosis	Total dose to improve (mg.)	Type of improvement	Duration of improvement (days)	Toxicity	Status—No. of days after onset of therapy
3 150 Kaposi's sarcoma	115	*Status quo*	37	Leukopenia in 23 days	Living—37
4 76 Osteogenic sarcoma	130	Decr. tumor size after 10 days	29	None	Dead—171
6 330 Chr. myel. leukemia —near acute phase	40	Decr. total WBC & size of spleen lasting 7 days. No change in rest of blood picture	7	None	Dead—66
7 1 Chr. myel. leukemia	180	Decr. WBC—520,000 to 31,000 starting in 5 days	28	None	Dead—18 mo.
11 165 Lymphosarcoma	70	Decr. abdominal masses in 7 days lasting 14 days, then relapse	14	Leukopenia in 28 days (after improvement)	Living—21
13 15 Hodgkin's disease	120	Decr. cough in 3 weeks. All nodes decr. in 5 weeks	135	Leukopenia in 18 days	Living—2 yr. mo.
14 5 Hodgkin's disease	145	All nodes decr. Gained weight after 28 days	120	Leukopenia in 21 days	Dead—1 yr. mo.
15 22 Hodgkin's disease	140	Complete remission. All nodes disappeared after 3 mo. therapy	1 yr. 8 mo.	Leukopenia in 38 days	Living—1 yr. 1 mo.
16 13 Hodgkin's disease	80	*Status quo* (Thio-TEPA given because of other drug shortage)	4	None	Living—1 yr. 1 mo.

TABLE 7 (*Continued*)

Case No. C.R. No. Diagnosis	Total dose to improve (mg.)	Type of improvement	Duration of improvement (days)	Toxicity	Status—No. of days after onset of therapy
17 6 Chr. lymph. leukemia	555	Decr. nodes, improved hematological picture after 2 months	195	None	Living—2 yr.
18 114 Chr. lymph. leukemia	120	Decr. nodes & improvement of blood picture after 10 days	42	None	Dead—60 Tbc pneum.
21 148 Ca. lung	100	*Status quo*	30	Leukopenia after 34 days	Living—34
23 448 Ca. lung	194	*Status quo.* Questionable decr. axillary node	150	Leukopenia in 25 days	Dead—1 yr. 1 mo.
28 387 Ca. hypopharynx	180	Decr. cervical nodes in 6 weeks	11	Leukopenia	Dead—surgical complication
29 82 Ca. nasopharynx	120	Decr. cervical nodes after 9 days. Last radiation dose 8½ mo. prior	34	Leukopenia in 25 days	Dead—82
32 441 Ca. stomach	120	Decr. size of liver. Decr. I.I. & decr. alk. phos. after 2 weeks	4	Leukopenia in 15 days	Dead—38
36 120 Ca. sigmoid	121	Disappearance pleural fluid & then *status quo.* Patient's last radiation dose 5 weeks prior	120	Leukopenia in 29 days	Living—127
37 153 Ca. rectum	72.5	*Status quo*	30	Leukopenia in 20 days	Living—32
38 158 Ca. sigmoid	60	*Status quo*	21	None	Living—21

Table 7 (*Continued*)

Case No. C.R. No. Diagnosis	Total dose to improve (mg.)	Type of improvement	Duration of improvement (days)	Toxicity	Status—No. of days after onset of therapy
43 57 Ca. kidney & ca. gallbladder	90	Decr. size left flank mass, decr. pain & decr. alk. phos. Improvement started after 13 days	41	Leukopenia in 81 days	Dead—107
45 11 Papillary adenoca. ovary	70	Decr. ascites after 11 days & then *status quo*	75	Leukopenia in 12 days	Dead—142
47 121 Adenoca. ovary	330	Disappearance of all palpable abdominal mass after 30 days	75	None	Living—110
49 140 Papillary ca. ovary	230	Decr. abdominal masses & ascites after 6 days	60	Leukopenia in 26 days	Living—68
50 443 Ca. uterus	180	*Status quo*	133	Leukopenia in 20 days	Lost to study—133
54 399 Ca. breast	180	Decr. size of nodes, 1° breast tumor & liver after 15 days	30	Leukopenia in 60 days after regression	Dead—74
64 8 Ca. breast	230	After 5½ mo. decr. size of some soft tissue lesions	120	Leukopenia in 14 days	Dead—10½ mo.
66 355 Ca. breast	80	*Status quo*	210	Leukopenia in 27 days	Living—216
67 56 Ca. breast	165	Disappearance axillary node, decr. ascites & edema after 8 days	85	Leukopenia in 117 days	Dead—136. Responded before toxicity
68 31 Ca. breast	180	Disappearance of some subcutaneous nodules after 5 weeks	120	Leukopenia in 49 days	Living—300
69 106 Ca. breast	330	Soft tissue ulceration healed after 37 days	56	Leukopenia in 81 days	Living—210

TABLE 7 (*Concluded*)

Case No. C.R. No. Diagnosis	Total dose to improve (mg.)	Type of improvement	Duration of improvement (days)	Toxicity	Status—No. of days after onset of therapy
72 38 Malignant melanoma	145	Disappearance of all subcutaneous nodules after 10 days	27	Leukopenia in 22 days	Dead—51
73 42 Malignant melanoma	160	Disappearance of inguinal masses after 7 days	26	Leukopenia in 25 days	Dead—98
76 52 Malignant melanoma	195	*Status quo* with questionable decr. of injected nodule	300	Leukopenia in 16 days	Dead—11 mo.
79 74 Malignant melanoma	100	Decr. inguinal masses & edema after 12 days	17	Leukopenia in 21 days	Dead—43
82 131 Malignant melanoma	280	Disappearance of 2 subcutaneous nodules	98	None	Living—135
84 155 Malignant melanoma	200	Decr. size of right flank mass after 18 days, but cerebral lesion same	30	None	Living—60
87 81 Undiagnosed tumor—spinal cord	290	Decr. spinal fluid protein. Decr. pain & incr. ability to move legs. Improvement occurred in 11 days	78	Leukopenia in 38 days	Dead—216
91 30 Metastatic carcinoma —1° site ?	270	After 1 mo. decr. I.I. then *status quo*	132	Leukopenia in 48 days	Dead—184
93 371 Metastatic carcinoma —1° site ?	170	Healing of ulcerated scalp tumor 1 mo. & softening of cervical nodes	42	Leukopenia in 27 days	Dead—1 yr. 2 mo.

TABLE 8
SUMMARY OF THIO-TEPA RESULTS

Number Name C.R. No.	Diagnosis Age & Sex	Prior therapy Last dose*	Ind. dose Total dose (mg.)	Duration ℞—days	Objective results	Duration of improvement	Status Grade
(1) C. T. No. 64	Rhabdomyosarcoma 55—M	—	30–40 I.M. & into tumor T.D. 100 mg.	3 times over 14 days	Tumors increased	—	Dead 75 days 0
(2) E. D. No. 95	Soft tissue tumor, type? 55—M	—	20–30 I.M. & into tumor T.D. 75 mg.	3 times over 12 days	Tumors increased	—	Dead 0
(3) I. N. No. 150	Kaposi's sarcoma 65—F	None	10–40 I.M. & into tumor T.D. 115 mg. to date	5 times over 37 days	No essential change to date	*Status quo* for 37 days	Living 37 days ±
(4) I. K. No. 76	Osteogenic sarcoma 39—F		10–40 I.M. & into tumor T.D. 250 mg.	9 times over 64 days	Temporary decrease in size of recurrent tumor mass lower jaw & cheek after 10 days	29 days after 130 mg.	Dead 171 days +
(5) L. D. No. 445	Multiple myeloma 66—F	None	5–15 I.M. T.D. 25 mg.	3 times over 20 days	No change during ℞. Developed stroke & died		Dead 25 days 0
(6) E. C. No. 330	Chr. myel. leukemia (approach. acute leuk.) 55—M	Radiation, 28 mo. TEPA, 7 days	10–40 I.M. T.D. 90 mg.	4 times over 28 days	Temp. decrease WBC & size of spleen starting after 3 weeks. No chg. Hb, RBC, plate. or blasts or GC	7 days after 40 mg.	Dead 66 days +

(7) C. A. No. 1	Chr. myel. leukemia 31—M	None	20–50 I.M. T.D. 320 mg.	11 times over 47 days	Temp. improv. in hematological picture. WBC decr. 520,000–31,000. Started 5 days after ℞ to imp.	28 days & ℞ changed for other reasons; 180 mg.	Dead 18 mo. +
(8) R. F. No. 402	Lymphosarcoma 37—M	Radiation, 4 mo. HN2, 2 mo.	5–40 I.M. & into tumor T.D. 285 mg.	17 times over 64 days	Some tumors grew, others regressed. Had rad. ℞ with TSPA		Dead 9 mo. 0
(9) M. P. No. 60	Lymphosarcoma 72—F	None	20–30 I.M. T.D. 50 mg.	2 times over 10 days	Disease not affected by ℞		Dead 43 days 0
(10) J. B. No. 2	Lymphosarcoma 60—M	Folic acid antag., 5 yr., 5 mo. TEM—complete remission for 3 yr., again 2 yr., 3 mo. CB 1348, 9 mo. Meticorten, 2 mo. Act. D, 8 days	20–50 I.M. T.D. 240 mg.	6 times over 46 days	At onset ℞ pt. resistant to TEM & CB 1348. Disease progressed under TSPA & changed to other ℞		Living to date 110 days 0
(11) J. D. No. 165	Lymphosarcoma 50—F	Radiation, 4 mo. HN2, 4 mo. (no effect) On Meticorten, 4 mo. before & during therapy	30–40 I.M. T.D. 70 mg.	2 times over 9 days	Marked decr. abd. masses starting 1 wk. after insti. of ℞. Continued 2 wk. then relapse. On Meticorten during TSPA	2 weeks after 70 mg.	Living to date +
(12) W. S. No. 152	Hodgkin's disease 28—M	Chemotherapy for 4 yr., 9 mo. 6-MP, 3 mo.	10–20 I.M. T.D. 80 mg.	6 times over 29 days	No change. Changed to other ℞	*Status quo* 29 days	Dead 1 yr., 7 mo. 0

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* Time between last dose of prior therapy and institution of Thio-TEPA.

TABLE 8 (*Continued*)

Number Name C.R. No.	Diagnosis Age & Sex	Prior therapy Last dose*	Ind. dose Total dose (mg.)	Duration ℞ days	Objective results	Duration of improvement	Status Grade
13 F. J. No. 15	Hodgkin's disease 31—M	Radiation, 10 yr. Chemotherapy, 10 mo.	5–20 I.M. & P.O. T.D. 265 mg.	Over 318 days	Decr. in cough after 3 wk. Decr. in nodes after 5 wk. ℞. Continued for 4 mo. then relapse	4½ mo. after 120 mg.	Living 2 yr., 4 mo. +
14 B. G. No. 5	Hodgkin's disease 18—F	TEPA, 1½ yr prior, ending 7 days before TSPA	5–20 I.M. & P.O. T.D. 780 mg.	86 times over 252 days	Sl. decr. nodes, gain in wt. After 28 days pt. had thrombocytopenia with hemorrhage during ℞	4 mo. after 145 mg.	Dead 1 yr., 1 mo. +
(15) N. H. No. 22	Hodgkin's disease 14—F	TEM, 2 yr., 4 mo.	20–40 I.M. T.D. 140 mg.	4 times over 143 days	Complete remission after 3 mo.	1 yr., 8 mo. to date 140 mg.	Living to date 1 yr., 11 mo. +
(16) A. P. No. 13	Hodgkin's disease 26—F	TEM & CB 1348, 7 days Radiation, 3 yr.	40 mg I.M. T.D. 80 mg.	2 times over 4 days	No change. TSPA given few days because of other drug shortage	*Status quo* 80 mg.	Living to date 1 yr., 11 mo. ±
(17) A. S. No. 6	Chr. lymph. leukemia 32—M	None	5–40 I.M. & P.O. T.D. 555 mg.	38 times over 28 days	Decr. nodes & impr. bld. picture. Started after 2 mo. ℞ changed	6½ mo. after 555 mg. ℞ changed	Living to date 2 yr. +
(18) D. M. No. 114	Chr. lymph. leukemia 78—M	None	20 I.M. T.D. 120 mg.	6 times over 60 days	Decr. nodes, impr. bld. pictures. After 10 days exp. with tbc pneumonia	6 weeks after 120 mg.	Dead 60 days +
(19) J. K.	Ca. ear 82—M	Radiation, 1 mo.	40 into tumor T.D. 40 mg.	Once 1 day	Expired day of first dose		Dead 1 day 0

(20) S. W. No. 59	Ca. lung 65—M	Radiation, 1 yr.	20 I.M. T.D. 20 mg.	1 day	Patient lost to study 4 days after ℞	—	Dead 1½ mo. 0
(21) J. M. No. 148	Ca. lung 45—M		10–20 I.M. T. D. 100 mg.	7 times over 27 days	No essential change since inst. of ℞	*Status quo* 1 mo. after 100 mg.	Living to date 34 days ±
(22) E. J. No. 437	Ca. lung 74—M	None	20–40 I.M. or into tumor T.D. 140 mg.	7 times over 50 days	Tumor incr. in size	—	Dead 52 days 0
(23) H. W. No. 448	Ca. lung 55—M	None	14–40 intrapleural or I.M. T.D. 194 mg.	6 times over 82 days	*Status quo.* Questionable sl. decr. axil. node	*Status quo* 5 mo. after 194 mg.	Dead 1 yr., 1 mo. ±
(24) A. W. No. 27	Ca. mouth 56—M	AminoN of P., 4 mo. Radiation ℞, 7 mo.	40 into tumor T.D. 120 mg.	3 times over 17 days	Tumor areas injected sloughed away	—	Dead 19 days 0
(25) E. H. No. 144	Ca. tongue 57—M	Radiation ℞, 1 mo.	40 into tumor T.D. 40 mg.	1 day	None. Expired suddenly 6 days later	—	Dead 6 days 0
(26) L. McC. No. 454	Ca. tongue 58—F	Radiation ℞, 3½ mo.	20 I.M. T.D. 20 mg.	1 day	None. Expired 3 days after ℞	—	Dead 3 days 0
(27) J. B. No. 433	Ca. hypopharynx 67—M	None	20 I.M. T.D. 120 mg.	6 times over 17 days	Progressed downhill	—	Dead 28 days 0
(28) C. S. No. 387	Ca. hypopharynx 45—M	None	T.D. 180 mg.	9 times over 36 days	Decr. cervical mass after 6 wk. Then note pt. operated & expired with surg. compl.	11 days	Dead +

* Time between last dose of prior therapy and institution of Thio-TEPA.

TABLE 8 (*Continued*)

Number Name C.R. No.	Diagnosis Age & Sex	Prior therapy Last dose*	Ind. dose Total dose (mg.)	Duration ℞—days	Objective results	Duration of improvement	Status Grade
(29) W. Y. W. No. 82	Ca. nasopharynx 60—M	Radiation ℞, 8½ mo.	20–40 into tumor T.D. 240 mg.	8 times over 65 days	Decr. size neck nodes (more decr. in those injected) started after 9 days	34 days after 120 mg.	Dead 82 days +
(30) F. W. No. 401	Ca. stomach 54—M	None	10 I.M. T.D. 90 mg.	9 times over 40 days	Progressed downhill	-	Dead 61 days 0
(31) A. P. No. 407	Ca. stomach 52—F		20–40 I.M. T.D. 80 mg.	3 times over 8 days	Disease progressed & patient deteriorated	-	Dead 74 days 0
(32) G. R. No. 441	Ca. stomach 56—F		30 I.M. T.D. 120 mg.	4 times over 30 days	Sl. decr. size of liver after 2 wk. associated with decr. I.I. & alk. phos., then deterioration	4 days after 120 mg.	Dead 38 days +
(33) S. G. No. 421	Ca. large bowel 57—F	None	10 I.M. T.D. 70 mg.	7 times over 19 days	Progressed downhill. Palp. abd. mass same but urinary output decr.		Dead 22 days 0
(34) V. R. No. 426	Ca. large bowel 64—F	None	10 I.M. T.D. 70 mg.	7 times over 14 days	Deterioration		Dead 24 days 0
(35) E. C. No. 112	Ca. sigmoid Ca. ovary ? 68—F	None	10–20 I.M. T.D. 70 mg.	6 times over 24 days	Intestinal obstr. & coma impr. temp. for 9 days then relapse		Dead 24 days 0
(36) M. S	Ca. sigmoid 49—F	Radiation ℞, 5 wk.	2.5–30 mg. I.M. T.D. 121 mg.	17 times over 127 days	Disappearance of chest fluid 2 wk. after ℞. GC then	4 mo. after 121 mg.	Living to date 4¼ mo.

(37) O. S. No. 153	Ca. rectum 55—M	None	2.5–20 mg. I.M. T.D. 72.5 mg.	6 times over 32 days	*Status quo* to date	*Status quo* 1 mo. to date after 72.5 mg.	Living 32 days to date +
(38) F. H. No. 158	Ca. sigmoid 66—M	None	10–30 I.M. T.D. 80 mg.	5 times over 31 days	*Status quo* to date	*Status quo* 31 days	Living 2 wk. to date ±
(39) R. M. No. 416	Ca. rectum 54—M	6-M.P., 24 days	10 I.M. T.D. 150 mg.	15 times over 53 days	Tumor in inguinal region incr. progressively in size & patient went downhill		Dead 53 days 0
(40) H. B. No. 21	Ca. rectum 68—F	Chemotherapy, 36 days	5–30 P.O. & I.M. T.D. 215 mg.	24 times over 240 days	Tumor incr. in size		Dead 1 yr., 2 mo. 0
(41) R. McM. No. 437	Ca. rectum 36—F	None	10–30 I.T. or I.M. T.D. 330 mg.	14 times over 166 days	Tumor masses incr. in size		Dead 219 days 0
(42) E. S. No. 414	Ca. pancreas 48—M	6-M.P., 8 days	10–40 I.M. & I.P. T.D. 470 mg.	19 times over 112 days	Decr. ascites & edema after 1 mo. Diuretics given. *Status quo* next 2 mo.	Relatively *status quo* 2 mo. after 320 mg.	Dead 127 days 0
(43) F. C. No. 51	Ca. gall bladder Ca. kidney 54—F	None	20–30 I.M. T.D. 170 mg.	8 times over 81 days	Decr. size left flank mass, decr. alk. phos., decr. pain after 13 days	41 days after 90 mg.	Dead 107 days +
(44) E. J. No. 409	Seminoma 36—M	Radiation ℞, 41 days (no effect)	20–40 I.M. T.D. 160 mg.	6 times over 14 days	Pulmonary & bone lesions incr.	—	Dead 82 days 0

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* Time between last dose of prior therapy and institution of Thio-TEPA.

TABLE 8 (*Continued*)

Number Name C.R. No	Diagnosis Age & Sex	Prior therapy Last dose*	Ind. dose Total dose (mg.)	Duration ℞—days	Objective results	Duration of improvement	Status Grade
(45) G. B. No. 11	Ca. ovary 37—F	Radiation ℞, 7 mo.	5–30 P.O., I.P., or I.M. T.D. 105 mg.	16 times over 119 days	Sl. decr. ascites 2½ mo. Started in 14 days	Decr. ascites 2½ mo. after 70 mg.	Dead 142 days ±
(46) S. A. No. 75	Pap. adenocarcinoma ovary 13—F	None	40 I.M. T.D. 80 mg.	Twice over 7 days	Expired after 19 days	—	Dead 19 days 0
(47) L. S. No. 121	Ca. adenocarcinoma ovary 75—F	None	20–25 I.M. T.D. 330 mg. to date	14 times over 133 days	Complete disappearance of palp. tumor after 30 days	2½ mo. to date after 330 mg.	Living to date +
(48) I. A. No. 128	Pap. ca. ovary 70—F	None	25–40 I.P. T.D. 120 mg.	4 times over 45 days	Patient in intestinal obstr. Obstr. unrelieved & expired	—	Dead 56 days 0
(49) C. DeL. No. 140	Pap. ca. ovary 47—F	Radiation ℞ (700 r), 8 days	20–40 I.M. T.D. 230 mg. to date	13 doses in 63 days	Decr. masses & ascites	2 mo. to date after 230 mg.	Living 63 days to date +
(50) M. T. No. 443	Ca. uterus 53—F	Radiation ℞, 10 days	5–30 I.M. T.D. 180 mg.	12 times over 120 days	*Status quo*	*Status quo* 133 days after 180 mg.	Lost to study 133 days ±
(51) S. B. No. 438	Ca. uterus 77—F	Radiation ℞, 2 yr.	10 I.M. T.D. 10 mg.	1 day	Expired 2 days after institution of therapy	—	Dead 2 days 0

(52) B. C. No. 449	Ca. cervix 57—F	None	30 into cervical mass T.D. 30 mg.	1 day	Progressed downhill & expired in 5 days		Dead 5 days 0
(53) W. M. W. No. 444	Ca. cervix 44—F	Radiation ℞, 2 mo.	30 40 I.M. T.D. 140 mg.	4 times over 26 days	Progressed downhill. Tumor increased		Dead 40 days 0
(54) M. W. No. 399	Ca. breast 31—F	None	10 I.M. (17 times), I.P. (once) T.D. 190 mg.	18 times over 73 days	Regression nodes & masses after 15 days lasted 30 days, then progressed downhill	1 mo. after 180 mg.	Dead 74 days +
(55) E. H. No. 394	Ca. breast 38—F	Radiation ℞, 72 days	10 I.M. T.D. 30 mg.	3 times over 19 days	Incr. tumor masses & deterioration in condition		Dead 19 days 0
(56) E. P. No. 400	Ca. breast 70—F	None	10 I.M. T.D. 30 mg.	3 times over 15 days	Downhill		Dead 15 days 0
(57) A. J. No. 420	Ca. breast 65—F	Radiation ℞, 11 mo.	10 I.M. T.D. 10 mg.	1 day	Expired day of institution of therapy	—	Dead 1 day 0
(58) E. G. No. 423	Ca. breast 50+—F	Radiation ℞, 1 yr. Hormone Premarin, 4 mo.	10 I.M. T.D. 10 mg.	1 day	Expired in 6 days	—	Dead 6 days 0
(59) R. DeS. No. 376	Ca. breast 50+—F	Radiation ℞, 1 yr. A-Methopterin, 8 days	10 20 I.M. T.D. 290 mg. (10–20 mg. locally on bandage)	Over 162 days	Disease progressed slowly	—	Dead 165 days 0

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* Time between last dose of prior therapy and institution of Thio-TEPA.

TABLE 8 (*Continued*)

Number Name C.R. No.	Diagnosis Age & Sex	Prior therapy Last dose*	Ind. dose Total dose (mg.)	Duration ℞—days	Objective results	Duration of improvement	Status Grade
(60) A. G. No. 422	Ca. breast 38—F	Radiation ℞, 8 mo.	10 I.M. T.D. 10 mg.	1 day	Patient deteriorated	—	Dead 20 days 0
(61) M. W. No. 425	Ca. breast 77—F	None	10 I.M. T.D. 20 mg.	2 times over 5 days	Progressed downhill	—	Dead 8 days 0
(62) E. F. No. 439	Ca. breast 57—F	None	10–20 I.M. T.D. 160 mg.	9 times over 71 days	Disease progressed	—	Dead 93 days 0
(63) S. W. No. 455	Ca. breast 69—F	None	10 I.M. T.D. 10 mg.	1 day	Expired in 3 days	—	Dead 3 days 0
(64) B. McC. No. 8	Ca. breast (duct cell) 41—F	Radiation ℞, 6 mo.	5–30 I.M., into tumor, P.O. T.D. 505 mg. (180 mg. I.M., 90 mg. into tumor, 235 mg. oral)	7 times I.M. 4 times I.T. 39 times oral over 257 days	No change during 1st $3\frac{1}{2}$ mo. on I.M. ℞. 2 mo. after intratumoral ℞, decr. in soft tissue tumor. Bone lesions stationary	4 mo. after 230 mg.	Dead $10\frac{1}{2}$ mo. +
(65) R. S. No. 442	Ca. breast 49—F	None	10–20 I.M. or into tumor T.D. 100 mg.	7 times over 143 days	Sl. decrease in edema rt. arm after 51 days. Edema incr. & tumor incr.		Dead 224 days 0
(66) C. G. No. 355	Ca. breast 68—F	None	10–20 I.M. or into tumor T.D. 80 mg.	5 times over 126 days	No change in tumor or condition during 7 mo. ℞. Pt. lost to study	7 mo. *status quo* after 80 mg.	Lost to study ±

(67) A. M. No. 56	Ca. breast 56—F	Radiation ℞, 6 mo. Hormone ℞, 7 mo. & for 44 days during TSPA	5–30 I.M. or into tumor T.D. 235 mg.	11 times I.M. & once I.T. over 134 days	Axillary node injected with TSPA disappeared in 8 days. Ascites, perif. edema disappeared. Patient relapsed before toxicity	85 days after 165 mg.	Dead 136 days +
(68) D. D. No. 31	Ca. breast 53—F	Radiation ℞, 9 mo. Hormone ℞, 8 mo. AminoN of P., 1 wk.	5–40 I.M. or into tumor T.D. 430 mg.	18 times over 133 days	Subcut. nodules inj. with TSPA disappeared 1 wk. after intranod. injec. but prim. breast tumors unchgd.	1 mo. after 180 mg.	Living 10 mo. to date +
(69) C. C. No. 106	Ca. breast 61—F	None	20–50 into tumor T.D. 540 mg.	16 times over 100 days	37 days after onset ℞ large ulcerated nipple area, left, healed. Rt. breast less red. Improv. without toxicity	56 days after 330 mg.	Living 7 mo. to date +
(70) G. V. No. 135	Ca. breast 50—F	—	5–40 I.M. or into tumor T.D. 180 mg.	9 times over 46 days	Progression of disease	—	Living 3 mo. to date 0
(71) P. D. No. 366	Malig. melanoma 76—M	TEPA, 3 days	10 I.M. T.D. 30 mg.	3 times over 7 days	Progression of disease	—	Dead 7 days 0
(72) R. S. No. 38	Malig. melanoma 40—F		15–40 into tumor & I.M., & I.P. T.D. 165 mg.	7 times over 48 days	Disappearance of all subcutaneous nodules after intratumoral injec. over 8 days. Nodules gone by 10 days. Note: Improv. occ. without toxicity	27 days after 145 mg.	Dead 51 days +
(73) I. B. No. 42	Malig. melanoma 69—F	None	10–40 I.M. T.D. 280 mg.	13 times over 98 days	Inguinal mass disappeared. Decr. started after 7 days & gone in 18 days	26 days after 160 mg.	Dead 98 days +

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* Time between last dose of prior therapy and institution of Thio-TEPA.

TABLE 8 (*Continued*)

Number Name C.R. No.	Diagnosis Age & Sex	Prior therapy Last dose*	Ind. dose Total dose (mg.)	Duration ℞—days	Objective results	Duration of improvement	Status Grade
(74) A. F. No. 47	Malig. melanoma 51—F	None	20–40 I.M. T.D. 240 mg.	9 doses over 73 days	Tumor progressed	—	Dead 130 days 0
(75) N. G. No. 48	Malig. melanoma 55—M	None	20–40 I.M. & into tumor T.D. 100 mg.	4 times over 11 days	Expired 12 days after ℞	—	Dead 12 days 0
(76) D. S. No. 52	Malig. melanoma 17—M	Radiation ℞, 2 weeks	5–30 I.M. or into tumor T.D. 345 mg. (90 into nodes & 255 I.M.)	24 times over 316 days	Sl. decr. cervical masses; then condition stationary	5 mo. *status quo* 195 mg.	Dead 11 mo. ±
(77) J. S. No. 67	Malig. melanoma 68—F	None	20–30 I.M. T.D. 150 mg.	7 times over 34 days	Disease progressed	—	Dead 34 days 0
(78) M. P. No. 71	Malig. melanoma 37—F		10–20 into tumor & I.M. T.D. 120 mg.	8 times over 96 days	Disease progressed	—	Dead 340 days 0
(79) J. M. No. 74	Malig. melanoma 56—M	None	10–40 I.M. T.D. 195 mg.	7 times over 42 days	Decr. size inguinal masses & edema of legs after 12 days	17 days after 100 mg.	Dead 43 days +
(80) C. C. No. 104	Malig. melanoma 73—F	Radiation ℞, 4 mo.	10–50 into tumor or I.M. T.D. 400 mg.	14 times (of these 2 I.M.) over 112 days	Disease progressed		Dead 117 days 0

W. S. No. 129	Malig. mela- noma 56—M	None	30-40 I.M. or I.P. T.D. 140 mg.	4 times over 45 days	Disease progressed		Dead 57 days 0
(82) B. H. No. 131	Malig. mela- noma 55—F	None	15-40 into tumor or I.M. T.D. 280 mg. to date	10 times over 106 days to date	2 subcut. nodules disappeared. Wt. gain, GC better. Impr. started 8 days after inst. of ℞, continued to impr. to max. 14 days later, then *status quo* 3 mo.	98 days after 280 mg. to date	Living 4½ mo. to date +
(83) C. G. No. 141	Malig. mela- noma 27—F	None	10-40 into tumor or I.M. T.D. 180 mg.	7 times (6 times into tumor & once I.M.) over 37 days	Disease progressed. Nodule injected remained same size		Dead 39 days 0
(84) J. L. No. 155	Malig. mela- noma 52—M	None	20-40 I.M. T.D. 200 mg. to date	6 times over 46 days	Decr. rt. flank subcut. mass 18 days after ℞. Drowsiness & vomiting unchanged	1 mo. to date 200 mg.	Living to date 2 mo. +
(85) H. R. No. 156	Malig. mela- noma 63—M	None	50 I.M. T.D. 50 mg.	1 day	Expired in 4 days	—	Dead 4 days 0
(86) E. S. No. 130	Malig. mela- noma 57—F	None	30-40 I.M. T.D. 235 mg.	7 times over 43 days	Subcut. nodules & liver & abd. mass unchanged during ℞. Developed cereb. metast.	—	Dead 75 days 0
(87) D. S. No. 81	Undiagnosed tumor of spinal cord 22—F	Radiation ℞, 2 mo.	20-60 intrathecally T.D. 350 mg.	10 times over 176 days	Decr. spinal fluid, protein from 1480 to 264 to 112 starting 11 days after inst. of ℞ assoc. with decr. back pain & incr. ability to move legs	78 days after 290 mg.	Dead 216 days +

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* Time between last dose of prior therapy and institution of Thio-TEPA.

TABLE 8 (*Concluded*)

Number Name C.R. No.	Diagnosis Age & Sex	Prior therapy Last dose*	Ind. dose Total dose (mg.)	Duration ℞—days	Objective results	Duration of improvement	Status Grade
88 M. B. P. No. 432	Metast. ca. prim. site ? 55—F	None	40 I.P. T.D. 120 mg.	3 times over 11 days	Progressed downhill. Disease increased	—	Dead 20 days 0
89 J. B. No. 436	Metast. ca. prim. site ? 44—M	None	10 30 I.M. T.D. 95 mg.	5 times over 50 days	Questionable decrease in size of liver after 1 mo.	—	Dead 80 days 0
(90) W. B. No. 424	Metast. ca. prim. site ? 57—M	A-Methopt. & 6 M.P., 12 days	30 I.P. T.D. 60 mg.	2 times over 6 days	Expired 7 days after ℞.	-	Dead 7 days 0
(91) A. S. No. 30	Metast. ca. prim. site ? 40—F		10 40 I.M. 5 mg. P.O T.D. 370 mg.	12 times I.M. over 4½ mo., P.O. 34 days. Total 173 days over 5¾ mo.	After 1 mo. decr. icteric index 91 to 40. GC unchanged first 132 days	4½ mo. after 270 mg.	Dead 184 days (6 mo.) ±
(92) R. S. No. 404	Metast. ca. prim. site ? 44—M		10 I.M. T.D. 10 mg.	1 day	Changed to other ℞	—	Dead 82 days 0
(93) B. H. No. 371	Metast. ca. prim. site ? 43—F	Radiation ℞, 1 yr., 2 mo.	5 30 I.M. T.D. 395 mg.	28 times over 231 days	After 1 mo. ulcerated tumor on scalp dried & crusted. Cervical nodes softer	6 weeks after 40 mg.	Dead 1 yr., 2 mo. +
(94) W. W. No. 50	Metast. ca. prim. site ? 60—M		10 20 I.M. & once 20 mg. I.P. T.D. 130 mg.	8 times over 46 days	Progressed downhill	—	Dead 111 days 0

One patient with a malignant melanoma with 11 subcutaneous nodules, ascites, and intestinal obstruction, was treated with Thio-TEPA injections into one of the subcutaneous nodules. Ten days after institution of therapy all of the subcutaneous nodules disappeared, although the partial intestinal obstruction persisted.

Table 7 shows an analysis of the cases showing objective improvement, questionable improvement, or *status quo* with Thio-TEPA therapy. Table 8 is a summary of data on the 94 cases in our series of Thio-TEPA-treated patients.

The duration of improvement or objective tumor regression displayed a high degree of variability, a characteristic already observed in the data on the amounts of the drug necessary to produce remission. The duration of tumor regression of the 27 patients ranged from 1 week to more than 1 year. Table 9 shows the duration of tumor regression of the 27 patients.

Table 9

Duration of Objective Improvement of 27 Cases Treated with Thio-TEPA

Weeks	Number of cases
1	1
2	2
3	2
4	2
5	5
6	1
7	2
8	0
9	2
10	0
11	1
12	1
Months	
3–5	6
5–7	1
7–12	0
More than 12	1

Of the 94 patients treated with Thio-TEPA, 74 are dead, 18 are living and under study, and 2 were lost to study.

Thirty-two patients in the series were treated for 21 days or less. Of these there were 6 with sarcomas or related diseases, one of whom showed temporary objective tumor regression and one who remained unchanged. There were 26 carcinomas; all progressed downhill.

There were 62 cases treated for periods over 21 days. Of the 12 cases of sarcoma and related disease, 8 showed tumor regression and 1 showed questionable improvement. Of the 50 cases of carcinoma, 18 showed regressions, 10 showed questionable improvement or a stationary condition, and in 22 cases the disease continued, apparently unaffected. Tables 10 and 11 show the results of Thio-TEPA therapy in the cases treated less than 21 days and those treated more than 21 days.

The development of toxicity did not appear necessary to achieve a beneficial effect from Thio-TEPA. Ten of the 39 patients who appeared to show

Table 10

Results of Cases Treated with Thio-TEPA 21 Days or Less

	Number of cases	Objective tumor regression	Questionable improvement—*status quo*	Downhill
Sarcomas & related diseases........	6	1	1	4
Carcinomas.....................	26	0	0	26
Total.........................	32	1	1	30

Table 11

Results of Cases Treated with Thio-TEPA Over 21 Days

	Number of cases	Objective tumor regression	Equivocal	Downhill
Sarcomas & related diseases............	12	8	1	3
Carcinomas.........................	50	18	10	22
Total.............................	62	26	11	25

a beneficial effect did not develop leukopenia during therapy. Four other cases experienced maximum improvement prior to toxicity.

Discussion

In this study our general principle in the administration of Thio-TEPA was to use it until the tumor regressed or the patient displayed mild toxicity. This, of course, is not a method of administration that should be advised for general therapy. It was used this way as an investigative tool to explore the maximum therapeutic effect of the drug and to be certain that the drug was not discarded as having little or no value before it had been used in sufficient doses over a sufficient period of time.

We used the intratumoral injections for a twofold purpose: (1) to produce the highest concentration in the accessible tumor area, and (2) to exert its effects also in distant areas of the body where tumor may have been present. That intratumoral administration does exert effects in distant areas is established by the facts that leukopenia developed following intratumoral injections, and that tumor masses distant to the site of injection were seen to regress.

Thio-TEPA can be used safely for long periods of time without any serious ill effect to the patient if certain precautions are observed. As yet, we have not had a fatality that can be attributed to this agent.

While the drug, as administered in our study, has not yielded such favorable results as others have reported, its freedom from dangerous or uncomfortable side effects, combined with the possibility of at least temporary relief makes us feel that it may well have at least a temporary place in the palliation of uncontrollable malignancy.

Conclusions

In conclusion, triethylene thiophosphoramide appears to exert a temporary beneficial effect in some cases of neoplastic diseases.

In our series of 94 patients, the diagnoses of those who showed moderate temporary tumor regression with Thio-TEPA included Hodgkin's disease, lymphosarcoma, chronic myelogenous leukemia, chronic lymphatic leukemia, carcinoma of the ovary, carcinoma of the breast, a spinal cord tumor, and malignant melanoma. Lesser improvement was observed in patients with other types of tumors.

A clear statement of the position of this compound in the armamentarium of cancer chemotherapeutic drugs available today awaits study of a larger series of adequately treated patients.

A clearer statement as to the place of Thio-TEPA in the palliation of neoplastic disease depends on the establishment of a clearer set of criteria as to what constitutes palliation.

Acknowledgment

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References

1. Farber, S., R. Toch, E. M. Sears & D. Pinkel. 1956. Advances in chemotherapy of cancer in man. Advances in Cancer Research. **4**: 1–71. J. P. Greenstein & A. Haddow, Eds.
2. Buckley, S. M., C. C. Stock, R. P. Parker, M. L. Crossley, E. Kuh & D. R. Seeger. 1951. Inhibition studies of some phosphoramides against sarcoma 180. Proc. Soc. Exptl. Biol. Med. **78**: 299–305.
3. Crossley, M. L., J. B. Allison, R. P. Parker, E. Kuh & D. R. Seeger. 1952. Chemotherapy of cancer in rats. IV. The treatment of transplanted cancers in rats with N-pentamethylene- and N-(3-oxapentamethylene)-N′,N″-diethylenephosphoramides. Cancer Research. **12**: 256.
4. Burchenal, J. H., S. F. Johnston, R. P. Parker, M. L. Crossley, E. Kuh & D. R. Seeger. 1952. Effects of the N-ethylene substituted phosphoramides on transplantable mouse leukemia. Cancer Research. **12**: 251–252.
5. Personeus, G., S. L. Halliday, D. McKenzie & J. H. Williams. 1952. Effect of a series of ethyleneimine derivatives against metastasizing mammary adeno carcinoma of the rat. Proc. Soc. Exptl. Biol. Med. **81**: 614–616.
6. Crossley, M. L., J. B. Allison, R. P. Parker, E. Kuh & D. R. Seeger. 1953. Chemotherapy of tumors in rats with certain ethylene phosphoramides. Proc. Soc. Exptl. Biol. Med. **83**: 438–447.
7. Sparks, S. J., M. L. Stevens, M. J. Landes, S. L. Halliday, D. McKenzie & J. H. Williams. 1953. The effect of a series of ethyleneimine derivatives on myeloid chloroleukemia in the rat. Blood. **8**: 653–660.
8. Shay, H., M. Gruenstein & J. Atwater. 1953. Study of effects of triethylene thiophosphoramide (Thio-TEPA) in two varieties of myelogenous leukemia in rats. Proc. Am. Assoc. Cancer Research. **1**(1): 50.
9. Shay, H., M. Gruenstein & J. Atwater. 1955. Effect of triethylene thiophosphoramide and 6-mercaptopurine in chronic myelogenous and in an acute leukemia in rats. Acta Unio Intern. contra Cancrum. **11**: 179–185.

10. Sugiura, K. 1954. The effect of thiotriethylene phosphoramide on the growth of a variety of mouse and rat tumors. Proc. Am. Assoc. Cancer Research. **1**(2): 47–48.
11. Sugiura, K. & C. C. Stock. 1955. Studies in a tumor spectrum. III. The effect of phosphoramide on the growth of a variety of mouse and rat tumors. Cancer Research. **15**: 38–51.
12. Farber, S., R. Appleton, V. Downing, F. Heald, J. King & R. Toch. 1953. Clinical studies on the carcinolytic action of triethylene phosphoramide. Cancer. **6**: 135–141.
13. Sykes, M. P., D. A. Karnofsky, F. S. Philips & J. H. Burchenal. 1953. Clinical studies on triethylene phosphoramides and diethylene phosphoramide compounds with nitrogen mustard-like activity. Cancer. **6**: 142–148.
14. Downing, V., S. Farber & M. Abu Hydar. 1953. Further studies on the effect of triethylene phosphoramides on malignant melanoma. Abstr. Proc. Am. Assoc. Cancer Research. **1**: 13.
15. Wright, J. C., A. Prigot, M. Logan & L. M. Hill. 1955. The effect of triethylene melamine and triethylene phosphoramide in human neoplastic diseases. Acta Unio Intern. contra Cancrum. **11**: 220–257.
16. Shay, H., C. Zarafonetis, N. Smith, I. Woldow & D. C. H. Sun. 1953. Treatment of leukemia with triethylene thiophosphoramide (Thio-TEPA): preliminary results in experimental and clinical leukemia. A.M.A. Arch. Internal Med. **92**: 628–645.
17. Shay, H. & D. C. H. Sun. 1955. Clinical studies of triethylene thiophosphoramide in the treatment of inoperable cancer. Cancer. **8**: 498–511.
18. Smith, N. J., S. Rosello, S. Chile & H. Shay. 1955. The effect of triethylene thiophosphoramide in the treatment of leukemia and certain lymphomas in infants and children. J. Pediat. **46**: 493–505.
19. Zarafonetis, C. J. D., H. Shay & D. C. H. Sun. 1955. Triethylene thiophosphoramide in the treatment of chronic leukemia. Cancer. **8**: 512–522.
20. Zarafonetis, C. J. D., H. Shay & D. C. H. Sun. 1955. Triethylene thiophosphoramide in the treatment of chronic leukemia. Acta Unio Intern. contra Cancrum. **11**: 332–347.
21. Bateman, J. C. 1955. Chemotherapy of solid tumors with tri-ethylene thiophosphoramide. New Engl. J. Med. **252**: 879–887.
22. Bateman, J. C. & H. N. Carlton. 1956. Palliation of mammary carcinoma wit phosphoramide drugs. J. Am. Med. Assoc. **162**: 701–706.
23. Bateman, J. C. & M. McCabe. 1955. Palliation of far-advanced cancer with tri ethylene thiophosphoramide. Acta Unio Intern. contra Cancrum. **11**: 111–114.
24. Bateman, J. C., B. Moulton & N. J. Larsen. 1955. Control of neoplastic effusio by phosphoramide chemotherapy. A.M.A. Arch. Internal Med. **95**: 713–719.
25. Bateman, J. C. & T. Winship. 1955. Treatment of human mammary carcinom with tri-ethylene thiophosphoramide and oxapentamethylene. Proc. Am. Assoc Cancer Research. **2**(1): 3.
26. Bateman, J. C. & T. Winship. 1956. Palliation of ovarian carcinoma with phos phoramide drugs. Surg. Gynecol. Obstet. **102**: 347–354.
27. Sykes, M. P., S. Philips & D. A. Karnofsky. 1956. Comparative therapeuti activity of the nitrogen mustards and allied compounds. Med. Clin. N. Am. **40** 837–856.
28. Ultmann, J. E., G. A. Hyman, C. Crandall, H. Naujoks & A. Gellhorn. Tr ethylene thiophosphoramide (Thio-TEPA) in the treatment of neoplastic diseas Unpublished data.
29. Wright, J. C. 1955. Chemotherapy of human cancer. Trans. N. Y. Acad. Sc **17**(3): 210–236.
30. Wright, J. C., P. Foster, B. Billow, S. L. Gumport & J. P. Cobb. 1958. Th effect of triethylene thiophosphoramide on 50 patients with incurable neoplast diseases. Cancer. To be published.

BUSULFAN (1,4-DIMETHANESULFONYLOXYBUTANE, MYLERAN): SUMMARY OF CLINICAL RESULTS

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The chief role of 1,4-dimethanesulfonyloxybutane (busulfan, Myleran) is in the treatment of chronic granulocytic leukemia. The compound is active in chronic lymphocytic leukemia but, because of its tendency to depress the platelet count, is not the drug of choice. In our hands it has not been effective in acute leukemia or in other forms of malignant disease, even when doses sufficient to cause severe bone marrow depression have been used.

Clinical Material

Forty-two patients have been treated for chronic granulocytic leukemia with busulfan and 3 with dimethyl Myleran. Twenty-one patients had received no previous treatment other than blood transfusion; 24 had received splenic irradiation (19 cases), radioactive phosphorus, urethan (2 cases each), or nitrogen mustard (1 case) and, in 2 cases, combinations of the several forms of treatment. Patients treated by radiotherapy received up to 11 courses (11 in 1 case, 4 and 3 in 3 cases, 2 in 2 cases, and 1 in 10 cases), and 1 required regular blood transfusions for more than a year.

Administration

Busulfan was given orally in 0.5 and 2 mg. tablets. Dimethyl Myleran was given in 1 mg. tablets. Three dosage schedules were used. Twelve of the first patients received doses of 1 to 3 mg. per kg. of body weight in 1 to 6 days (100 to 150 mg.). Three patients suffered severe bone marrow depression and the method was therefore abandoned. In 31 cases the daily dose was 4 mg. (approximately 60 γ per kg. of body weight). For dimethyl Myleran the daily dose was 2 to 2.5 mg. Treatment was continued until optimal clinical benefit was obtained 3 to 7 months later. This proved to be the most useful dose schedule. An intermediate dose range (8 and 10 mg. daily) was used in 2 cases. Twenty-one patients received 1 course of busulfan, but repeated courses were given in 11 cases (4 in 1 case, 3 in 4, and 2 in 6 cases). The indications for retreatment have varied. Earlier in the trial, when it was thought desirable to use the drug sparingly, treatment was deferred until symptoms reappeared. More recently, however, treatment has been resumed earlier in the hope of preventing clinical relapse.

Maintenance therapy. Intermittent therapy was found to have certain disadvantages. Most untreated patients remained well for many months after their first course of busulfan, but most of those who had already received much treatment, whether by radiotherapy or busulfan, relapsed quickly when the drug was discontinued. Maintenance therapy was therefore attempted. Twenty-five patients have been so treated; 17 patients had already received radiotherapy or busulfan (1 to 3 courses of radiotherapy in 8 cases, 1 to 3 courses of busulfan in 6 cases, and both radiotherapy and busulfan in 3 cases), and 8 patients had not been treated.

There were two types of cases in which maintenance therapy was indicated. If the leukocyte count fell unusually slowly during daily treatment with 4 mg. of busulfan, administration was continued indefinitely. In such cases the hemoglobin increase was often as rapid as in cases where the leukocyte count fell more quickly, and there seemed no good reason for increasing the dose. In other cases, stopping busulfan led to rapid increase in leukocyte count, and maintenance therapy was used in an effort to achieve stability. In such cases daily doses of 0.5 to 2 mg. sufficed to maintain a stable blood picture for several months. It was found necessary every few months to increase the daily maintenance dose by increments of 0.5 mg. to keep the leukocyte count steady; patients entering their fourth year of maintenance therapy required up to 4.5 mg. (in 1 case) of busulfan daily. In a few patients, 0.5 mg. daily led to reduction in leukocyte count to 5 to 6000 per cu. mm. when treatment was stopped. The duration of maintenance therapy ranged from 8 to 45 months, was over 24 months in 8 cases and over 36 months in 3.

Side Effects

Permanent amenorrhea occurred in all 4 premenopausal patients. Three female patients acquired temporary pigmentation of the skin, and 2 men developed mild gynecomastia. Hyperuricemia has not occurred.[1]

Results

Only one patient, a deaf mute, failed to respond in some degree to busulfan; it is not certain that she took her tablets. She later responded well to radiotherapy. Excluding two patients in myeloblastic relapse whose response, although dramatic, was fleeting, the median survival from the time of diagnosis in the 32 deceased patients in whom busulfan was used alone or following other forms of therapy was 36 months, compared with 26 months in a group of 33 patients treated only by radiotherapy before 1950. The mean survival was 35.5 months, compared with 28.8 months for the X-ray treated group. The median survival from the time of diagnosis of 15 deceased patients treated by busulfan alone was 31 months, compared with 38 months in the 17 deceased patients who received both radiotherapy and busulfan. The mean survivals were 27.6 months and 40 months (FIGURE 1).

Twelve patients died within 1 year of starting busulfan therapy. Two were treated in myeloblastic relapse, and 1 died of a cardiovascular accident during remission. Six of the 9 remaining patients received treatment with busulfan by methods that would not now be acceptable. Six patients responded initially but became progressively resistant after the first course 2 died of drug-induced pancytopenia and, in 1 case, the cause of death was not established. In 1 pancytopenic case, large doses of busulfan were given in the other, 4 mg. daily was continued after the leukocyte count had fallen below 10,000 per cu. mm., the patient having failed to attend the hospital Seven of these 12 patients had received previous treatment; 1 had no responded to radiotherapy to the spleen (2700 r in 11 weeks); 1 had no responded to urethan, 5 had received 2, 3, 3, 4, and 4 courses, respectively, of radiotherapy. In this group the median survival from the time of diagnosis was 18 months, and the mean survival was 21.8 months.

Maintenance therapy. The median survival from the time of diagnosis in the 25 patients treated with continuous busulfan was 37 months, and the mean survival was 40 months. These figures include the 6 living patients whose diagnosis was established 45, 40, 29, 27, 23, and 20 months ago, respectively. Of the 19 deceased patients, 2 died of intercurrent disease while under control; 10 slowly developed massive splenomegaly, although their hematological state was well controlled until myeloblastosis supervened; 7 became anemic or entered the myeloblastic phase without notable splenomegaly. In these 17 cases manifestations of relapse could not be checked by increasing the maintenance dose of the drug.

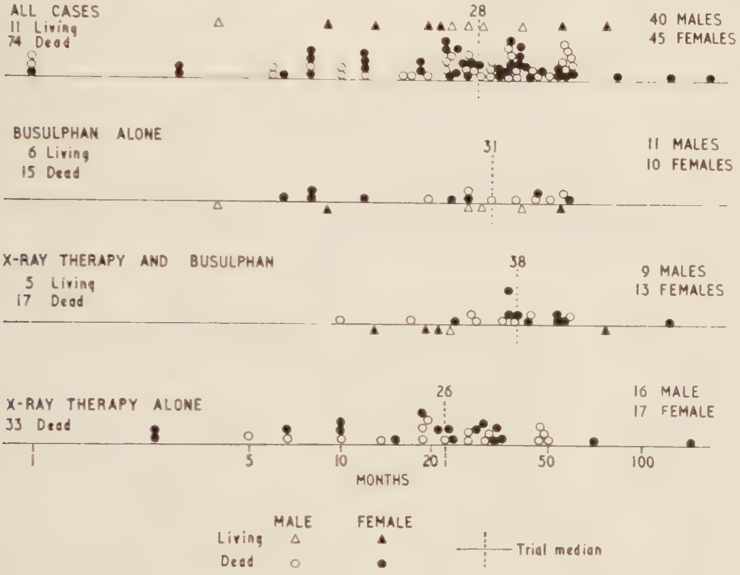

FIGURE 1. Chronic granulocytic leukemia in 85 patients: survival from diagnosis related to the type of therapy.

Dimethyl Myleran

Three patients have been treated with dimethyl Myleran. One had received a course of splenic irradiation 8 months previously (FIGURE 2). The daily doses were 2 to 2.5 mg. There were no side effects, and the clinical and hematological response in each case was identical with that expected if busulfan had been used in equivalent doses (FIGURES 2 AND 3).

Discussion

Busulfan is now an established form of therapy in chronic granulocytic leukemia.[1-11]. Frost and Jackson,[1] Haut *et al.*,[6] and Kurrle[9] prefer smaller

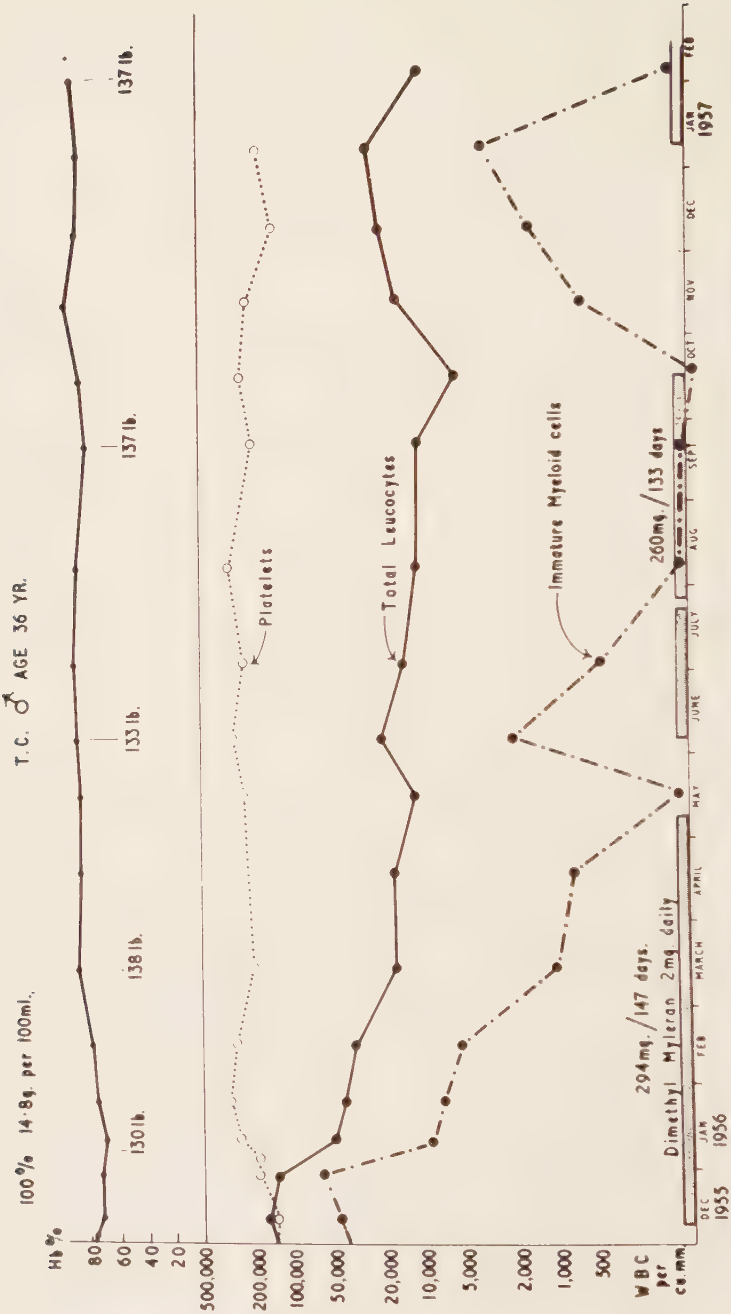

FIGURE 2. Chronic granulocytic leukemia: effect of dimethyl Myleran therapy on blood counts.

doses; Petrakis *et al.*[10] prefer short courses with larger doses; and Hyman and Gellhorn[1] favor intermediate dose ranges and have described in detail the precautions necessary for avoiding irreversible bone marrow damage. Busulfan is as effective as radiotherapy, but our survival figures, although inadequate, suggest that it is not superior to radiotherapy in its effect on survival; it is possible that radiotherapy and busulfan, used consecutively, will increase survival by a few months.

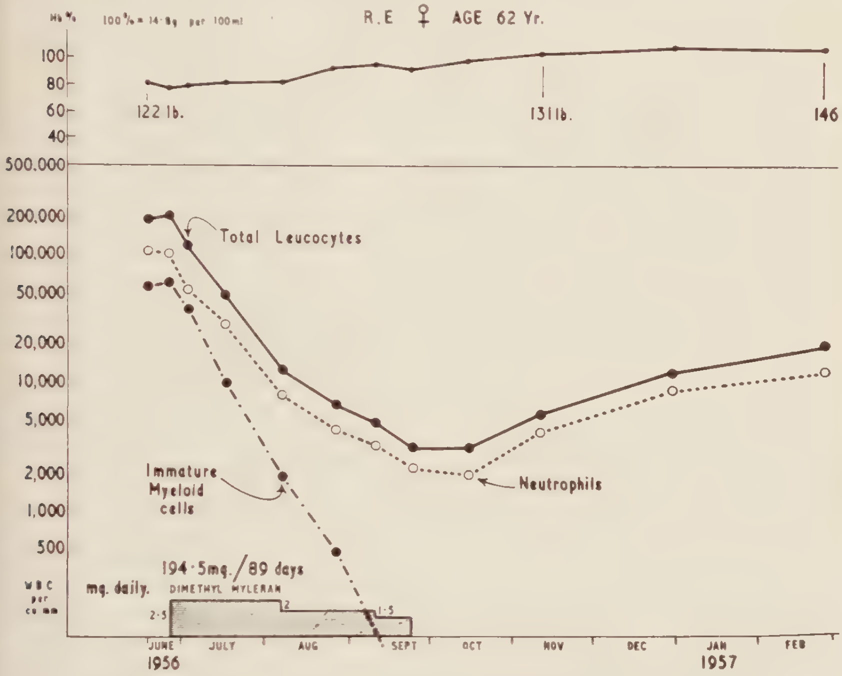

FIGURE 3. Chronic granulocytic leukemia: effect of dimethyl Myleran therapy on blood counts.

There is a wide range of sensitivity to the drug, but in this series only one patient appeared resistant and may not have taken the tablets. Thrombocytopenia was always a result of excessive dosage, but J. L. Stafford,[12] using 4 mg. daily, induced severe thrombocytopenia twice in one patient and had to abandon attempts at therapy. Busulfan is essentially a powerful depressant of hemopoietic tissue, and the preferential effect on granulopoietic cells is operative only at low dosage. With increasing doses, megakaryocytes and erythropoietic elements are affected and, in some of our patients treated with large doses (20 mg. daily), the initial rise in hemoglobin was followed by a period of relative depression between 5 and 8 months later. This did not occur when smaller doses were used. It would seem desirable to treat patients with the smallest amount of busulfan capable of achieving optimal clinical and hematological benefit for as long as possible. Whether it is the total dose consumed or the size of individual doses that ought to be kept as

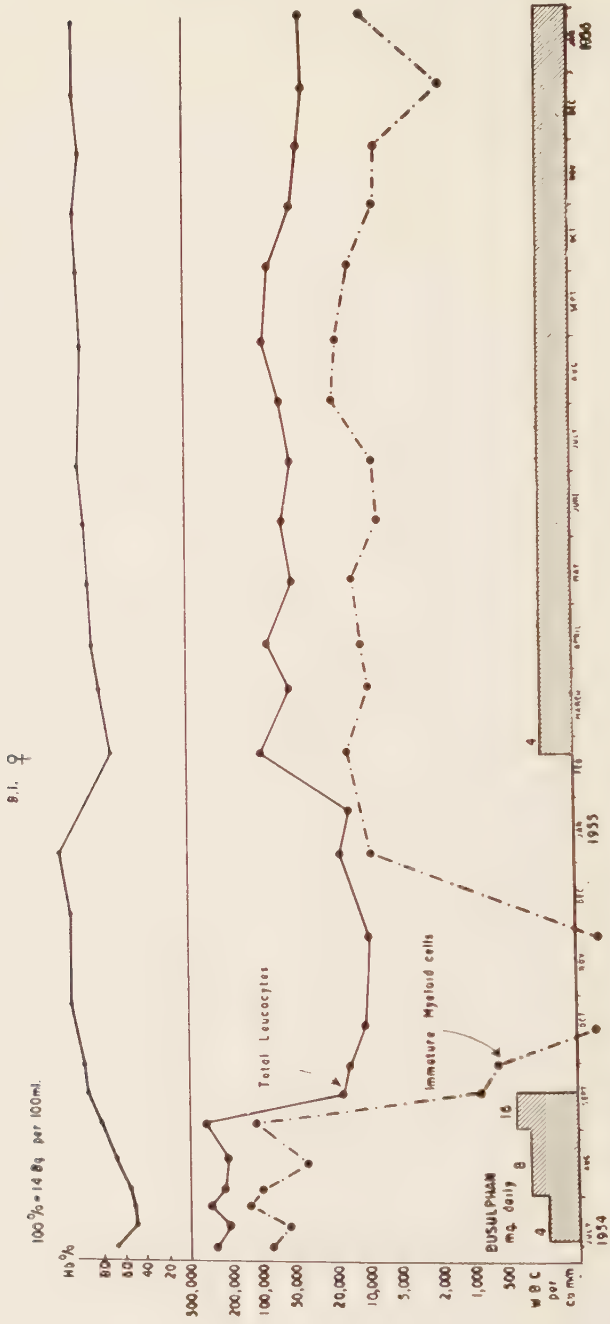

FIGURE 4. Chronic granulocytic leukemia: effect of busulfan therapy on blood counts. Large doses were required initially and later for maintenance.

low as possible is debatable. In the former case, maintenance therapy would be contraindicated. In practice, no patient receiving maintenance therapy has shown evidence of bone marrow depression. By analogy with antibiotics, it might be thought that long-continued treatment with small doses would lead frequently to drug resistance. Every patient does become progressively resistant to busulfan, just as to other forms of treatment, but this does not occur earlier with continuous than with interrupted therapy. With regard to the dosage used for the initial treatment, there is no doubt that splenic regression occurs more quickly with larger doses, but there is no improvement in the time required for the relief of symptoms and the increase in the rate of hemoglobin. This is true even when the leukocyte count falls very slowly (FIGURE 4). However, the best-maintained hemoglobin levels have been associated with leukocyte counts brought close to 10,000 per cu. mm. and kept there. If, therefore, after an initial period of 4 weeks on 60 γ per kg. of body weight per day the leukocyte count has not changed significantly, we should now double the dose and the frequency of performing blood counts. When doses of 60 γ per kg. of body weight per day are used it is rarely necessary to see the patient more frequently than once in 2 weeks; with higher doses, the rate of fall in leukocyte count is such that it is essential to see the patient and to perform blood counts more frequently.[1] This is a major consideration in treating a disease with a limited life span in which the aim is to help the patient to live as normal a life as possible. Patients receiving continuous therapy do not need to visit the hospital more often than once a month and, during well-controlled periods, two monthly visits have been sufficient.

Summary

The results of treatment with busulfan in 42 patients suffering from chronic granulocytic leukemia are summarized. Dimethyl Myleran was used in 3 additional cases. In 25 cases maintenance therapy was given. The results are discussed.

References

1. HYMAN, G. A. & A. GELLHORN. 1956. J. Am. Med. Assoc. **161**: 844.
2. BERNARD, J., G. MATHÉ & Y. NAJEAN. 1955. Semaine hôp. **31**(59): 3082–3087.
3. BLACKBURN, E. K., G. M. KING & H. T. SWAN. 1956. Brit. Med. J. **1**: 835.
4. FROST, J. W. & C. B. JACKSON. 1956. J. Am. Med. Assoc. **161**: 54.
5. GALTON, D. A. G. & M. TILL. 1955. Lancet. **1**: 425.
6. HAUT, A., S. J. ALTMAN, G. E. CARTWRIGHT & M. M. WINTROBE. 1955. A.M.A. Arch. Internal Med. **96**: 451.
7. INTROZZI, P. & G. MARINONI. 1955. Bibliographia Haematolgica. **21**(2): 22.
8. KLIMA, R., J. BEYREDER & E. HERZOG. 1954. Wien. klin. Wochschr. **35**: 682.
9. KURRLE, G. R. 1955. Med. J. Australia. **42**: 636.
10. PETRAKIS, N. L., H. R. BIERMAN, K. H. KELLY, L. P. WHITE & M. B. SHIMKIN. 1954. Cancer. **7**: 383.
11. PRIBILLA, W. & G. STOLLBERG. 1955. Deut. med. Wochschr. **80**: 1027.
12. STAFFORD, J. L. 1956. Personal communication.

CLINICAL INDICATIONS FOR THE USE OF NITROGEN MUSTARD AND TRIETHYLENE MELAMINE IN MALIGNANT LYMPHOMAS AND LEUKEMIAS

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A new era of cancer chemotherapy made its auspicious entry into the realm of clinical medicine with the introduction first, in 1946, of methyl-bis(β-chloroethyl)amine hydrochloride (HN2) and second, in 1951, of triethylene melamine (TEM) in the treatment of patients with Hodgkin's disease, lymphosarcoma, reticulum cell sarcoma, mycosis fungoides, and chronic lymphatic leukemia. Considerable clinical experience has accrued concerning the use of these compounds, especially in treating malignant lymphomas and chronic lymphatic leukemia.[1-7]

These polyfunctional alkylating compounds have been demonstrated to effect histological changes in cells identical to those of ionizing radiations. Certainly most, if not all, of the clinical and toxic manifestations of ionizing radiations have been mimicked, as well. For these reasons, these new agents and their congeners have been alluded to as radiomimetic compounds.

General Considerations

Primarily, HN2 and TEM are agents that have *acute* effects; that is, they render their effects rapidly, and frequently drastically, but for brief periods of time. In contrast, therapeutic X rays render *chronic* effects; that is, they work slowly and less dramatically through cumulative activity, but fre quently have a longer-lasting beneficial effect. The status of the activity o bone marrow may be a limiting factor in the use of these new agents, sinc they are acutely noxious to the cellular elements of the marrow. In only desperate clinical situations is it justifiable to employ HN2 or TEM whe the marrow is hypoplastic. Bulky nodal and visceral tumefaction respon transiently to these chemicals and less satisfactorily than they respond t X-ray therapy. Although pain due to bony involvement by these tumor may respond to treatment with HN2 and TEM, the response is usuall evanescent and unaccompanied by evidence of any degree of dissolution the bony tumor or of repair of the roentgen defects caused by the tumors Contrariwise, therapeutic X rays not only will often relieve pain, but als will allow affected and involved areas of bone to repair, as manifested b roentgen studies. Where, then, lie the clinical attributes of HN2 and TEM

Specific Considerations

HN2 and TEM are useful indeed in the following clinical settings: (1) clin ical Class II and III disease (TABLE 1); (2) emergency situations, such superior vena caval obstruction syndrome, spinal cord compression syndrom and central nervous system involvement with increased intracranial pressure; (3) acute toxic syndrome; (4) diagnostic dilemma; (5) *homme-rouge* type of mycosis fungoides; (6) the case with airway obstruction; and (7) the patient with widespread skin involvement by lymphoma or chronic lymphatic leukemia.

Table 1

Clinical Classification of Lymphomas

Class I. Disease limited clinically to a single locus (unifocal, unicentric origin); no constitutional symptoms or signs (that is, fever, night sweats, pruritus, weight loss, anemia, fatigue).

Class II. Disease limited regionally (regional anatomic distribution, that is, all apparent disease above the diaphragm, or all apparent disease below the diaphragm) with or without constitutional symptoms and signs.

Class III. Generalized (universal) disease with constitutional symptoms and signs.[9]

It is our contention that HN2 and TEM should not be used to treat patients with Hodgkin's disease, lymphosarcoma, or reticulum cell sarcoma who have clinical Class I disease. Patients with clinical Class II disease with constitutional symptoms and signs are candidates for treatment with these agents. Patients who fall into the clinical Class III category are almost always subjected one or more times to therapy with these compounds (Table 1).[9] In recent years we have administered HN2 in a single dose intravenously as 0.4 mg. per kg. of body weight whenever the bone marrow was demonstrated to be normocellular or hypercellular. The patients so treated recovered quickly from the induced nausea and vomiting that occurred usually within 12 hours, sparing them the daily harassment of these symptoms as rendered by the divided-dose technique. Cases of leukopenia and thrombocytopenia, although appearing more rapidly, were not more profound than those seen induced by the fractionated administration of HN2, nor more prolonged. Bone marrow and peripheral blood recovery from this insult occurred within 4 to 6 weeks.

HN2 and TEM represent an effective and rapid way to treat the patient with superior vena caval obstruction syndrome, the spinal cord compression complex, and central nervous system involvement.

In each of these clinical situations it is important to avoid active congestion in the tumor that might be effected by therapeutic X rays, with further compression ensuing. HN2 and TEM differ in this respect from X rays. They induce tumor regression without active congestion and swelling of the tumor, and obviate the possibility of further choking-off of a compressed spinal cord, obstructed great vessels, or compressed brain.

TEM causes less nausea and vomiting than HN2. It may be used interchangeably intravenously with HN2. The fractionated dose of TEM, when administered intravenously, is 0.04 mg. per kg. of body weight, giving this amount for three doses. A total dose of 0.12 mg. per kg. can be administered intravenously as a single injection, as the equivalent of 0.4 mg. per kg. of HN2. Therapeutically, however, TEM offers no advantage over HN2. This applies to treatment of the superior vena caval obstruction syndrome and the spinal cord compression complex. When there is clinical and laboratory evidence of increased intracranial pressure, such as that due to cerebral lymphoma or chronic lymphatic leukemia, it is perhaps wiser to employ TEM rather than HN2 in an attempt to obviate further increase of intracerebral pressure due to the vomiting subsequent to HN2 administration.

In every instance where HN2 or TEM is employed for the treatment of the superior vena caval obstruction syndrome, spinal cord compression syndrome, or central nervous system involvement by lymphoma or chronic lymphatic leukemia, X-ray treatment should be begun *immediately* thereafter. The latter must be given to the mediastinal mass in the case of superior vena caval obstruction, to the appropriate level of spinal cord compression, and to the brain and brain stem in the instance of central nervous system involvement.

Remarkable relief of symptoms and signs in these three states may be observed within 24 to 48 hours after administration of HN2 or TEM. In the caval syndrome, diuresis is noted frequently. The sequence of treatment whenever combination therapy is contemplated should be HN2 or TEM first, followed by X-ray therapy.

Recent studies revealed a 90 per cent recovery rate from paraplegia due to Hodgkin's disease in the epidural space in patients treated by a combination of HN2 and X-ray therapy.[10, 11]

The patient who is acutely ill with high fever, chills, and night sweats, with or without evidence of bulky or even palpable lymphomatous disease, is a good candidate for HN2 or TEM therapy. Rapid "detoxification" with relief of constitutional symptoms can be achieved usually within 1 to 3 days. Again, however, the remission may be short-lived unless reinforced by ancillary treatment with X rays. Pruritis, an enigma as concerns treatment, when present in patients with Hodgkin's disease is probably ameliorated more satisfactorily by HN2 or TEM than by any other method of treatment, albeit usually only temporarily.

Diagnostically and therapeutically, HN2 and TEM may serve as unique tools in the patient with known lymphoma who has had repeated pulmonary or mediastinal X-ray therapy, and who presents again with a pulmonary or mediastinal density on roentgen examination. Confronted with the diagnostic possibilities of atelectasis, recurrent tumor, a second primary tumor, or pulmonary fibrosis due to radiation, one can avoid X-ray therapy, and thus avoid the possibility of "compounding the felony," so to speak, should the problem be radiation fibrosis. HN2 or TEM given in such a situation could well clarify the setting since, if the mass were altered, it could then be explained best as lymphomatous disease with or without compression atelectasis. If it were not modified, then at least no further fibrosis would be induced.

Widespread cutaneous Hodgkin's disease, lymphosarcoma, reticulum cell sarcoma, or leukemia (lymphatic) cutis are clinical situations amenable to treatment with systemic agents such as HN2 and TEM. Frequently, flattening, shrinkage, and deflorescence of the lesions are achieved. Usually, residual pigmentation of the lesions ensues. Remission, although of variable length, is ordinarily short-lived. Perhaps the most successful treatment with these agents has been observed in patients with the *homme-rouge* type of mycosis fungoides. One such patient remained in complete remission for two years after a single full course of HN2!

Patients with lymphomas and chronic lymphatic leukemia not infrequently have involvement of the tonsils and nasopharynx to such a degree as to pose the problem of closure of the airway. Such patients can be treated effectively with HN2 or TEM (intravenously) without the hazard of active congestion in the tumors causing complete obstruction of the upper air passages. Radiation therapy, however, must be given soon thereafter in order to maintain the remission.

While HN2 must be given intravenously, TEM can be administered orally. Because of this virtue, TEM is singularly useful for the ambulatory management of patients with lymphomas and chronic lymphatic leukemia. Maintenance therapy also can be practiced with TEM. In patients with Hodgkin's disease, we give 10 mg. of TEM per week for 2 weeks, and then usually observe the patient for clinical and hematological effects in the third week. We do not exceed 40 mg. in any 1-month period. We divide the dose so that 2.5 mg. is taken daily for 4 successive days each morning, with water only, 1 hour preprandially. Alkali is not prescribed.

Patients with chronic lymphatic leukemia and lymphosarcoma are unusually sensitive to the effects of TEM. For this reason we proceed more conservatively and cautiously in these patients. Rapid and profound thrombocytopenia and leukopenia,[1] as well as overwhelming hyperuricemia may ensue in these patients. These patients are given 2.5 mg. × 1 or 2.5 mg. × 2 of TEM, and their clinical and hematological data are carefully observed for a week to 10 days before repeating the dosage. Once remission has been induced, then attempts at maintenance therapy are made using doses that, only by trial and error, show effectiveness of control of the disease in any single patient with lymphoma or chronic lymphatic leukemia.

General Comments

Although HN2 and TEM are important compounds in the treatment of these diseases, they manifest several pertinent shortcomings. With each successive course of HN2 or TEM the clinical tumor response is less marked and shorter-lived. In due time, either as a natural consequence of the disease or by virtue of repeated therapy, the patients have varying degrees of pancytopenia and bone marrow depression. On the whole, remissions achieved in these patients by HN2 and TEM have an average of a few months. HN2 frequently causes thrombophlebitis, despite various improved techniques in administration. It also causes nausea and vomiting in most patients. TEM is somewhat less predictable than HN2 in its marrow depressive effects and on the platelets and lymphocytes of the peripheral blood, but is much more potent on these same areas in patients with lymphosarcoma or chronic lymphatic leukemia. The poor performance of HN2 and TEM on bulky tumors and bone lesions in these patients is quite disappointing.

The advent of the newer polyfunctional alkylating compounds such as CB 1348 and Myleran,[12, 13] even though these agents have been unaccompanied ordinarily by nausea and vomiting, has not solved many of the inherent deficiencies displayed by HN2 and TEM in the management of patients with lymphoma or chronic lymphatic leukemia. Another disadvantage of these newer compounds, despite the facility of oral administration, is the interval of time necessary for cumulative action, usually 2 to 3 weeks. The seriously ill patient cannot be temporized with for this period of time.

Conclusions

Since 1946 with HN2, and since 1951 with TEM, a massive clinical experience has taken place with the employment of these antitumor radiomimetic compounds in the treatment of patients with malignant lymphomas and chronic lymphatic leukemia. The essential roles of these agents with regard to the clinical indications, dosage, toxicity, and results of treatment have been delineated. The shortcomings of these and the newer polyfunctional alkylating chemical materials have been expressed. Despite these objections, and because of their impact on clinical medicine and oncologic therapy, research and consequent clinical application of these compounds and their research offspring must continue if the fundamental knowledge of cancer and its treatment is to be developed.

References

1. Gilman, A. & F. S. Philips. 1946. Biological actions and therapeutic applications of β-chloroethyl amines and sulphides. Science. **103**: 409.
2. Rhoads, C. P. 1946. Nitrogen mustards in treatment of neoplastic disease. J. Am. Med. Assoc. **131**: 656.
3. Goodman, L. S., M. M. Wintrobe, W. Dameshek, M. J. Goodman, A. Gilman & M. T. McLennan. 1946. Nitrogen mustard therapy. Use of methyl-*bis*-(β-chloroethyl) amine hydrochloride and *tris*-(β-chloroethyl) amine hydrochloride for Hodgkin's disease, lymphosarcoma, leukemia, and certain allied and miscellaneous disorders. J. Am. Med. Assoc. **132**: 126.
4. Jacobson, L. O., C. L. Spurr, E. S. Guzman-Barron, T. R. Smith, C. Lushbaugh & G. F. Dick. 1946. Studies on the effect of methyl-*bis*(β-chloroethyl) amine hydrochloride on neoplastic diseases and allied disorders of the hemopoietic system. J. Am. Med. Assoc. **132**: 263.
5. Karnofsky, D. A., J. H. Burchenal, G. C. Armistead, C. M. Southam, J. L. Bernstein, L. F. Craver & C. P. Rhoads. 1951. Triethylene melamine in the treatment of neoplastic disease. A.M.A. Arch. Internal Med. **87**: 477.
6. Kravitz, S. C., H. D. Diamond & L. F. Craver. 1952. The clinical use of triethylene melamine. Blood. **7**: 729.
7. Rundles, R. W. & W. B. Barton. 1952. Triethylene melamine in treatment of neoplastic diseases. Blood. **7**: 483.
8. Spitz, S. 1948. The histological effects of nitrogen mustards on human tumors and tissues. Cancer. **1**: 383.
9. Diamond, H. D. 1953. Recent advances in the management of lymphomas and leukemias. Med. Clin. N. Am. **37**: 843.
10. Diamond, H. D. 1957. Hodgkin's disease: neurologic sequelae. Missouri Med. **54**: 945.
11. Williams, H., H. D. Diamond & L. F. Craver. 1958. On the neurological complications of the malignant lymphomas, leukemias, and allied diseases. Cancer. **11**: 76-82.
12. Galton, D. A. G. 1953. Myleran in chronic myeloid leukemia. Lancet. **264**: 208.
13. Galton, D. A. G., L. G. Israels, J. D. N. Nabarro & M. Till. 1955. Clinical trials of p-(di-2' chloroethylamino) phenylbutyric acid (CB 1348) in malignant lymphoma. Brit. Med. J. **2**: 1172.

CLINICAL EVALUATION OF CB 1348 IN LEUKEMIAS AND LYMPHOMAS*

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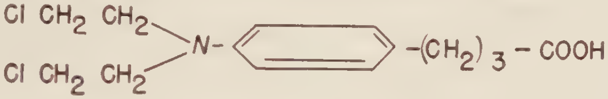
During the past ten years a large series of derivatives of nitrogen mustard have been synthesized at the Chester Beatty Research Institute in London, England.[1] One of these compounds is *p*-bis(2-chloroethyl)aminophenylbutyric acid (CB 1348)†. Its chemical structure is shown in FIGURE 1.



P-(di-2 CHLOROETHYLAMINO)-PHENYLBUTYRIC ACID; CB 1348,"CHLORAMBUCIL","LEUKERAN".

FIGURE 1

This compound, when administered shortly after implantation, is capable of producing complete inhibition of the growth of the Walker rat tumor.[1] It has been reported also to inhibit the lymphocytes in the peripheral blood of the rat, causing a rapid fall, while having no significant effect on the granulocytes.[2] These experimental results in animals have stimulated the clinical trial of this compound in diseases involving the reticuloendothelial system.

TABLE 1

DIAGNOSIS AND NUMBER OF PATIENTS TREATED WITH CB 1348

Hodgkin's disease	47
Chronic lymphocytic leukemia	18
Monocytic leukemia	16
Lymphosarcoma	8
Reticulum cell sarcoma	5
Lymphocytic lymphoma without leukemia (Cohnheim)	3
Acute lymphocytic leukemia	1
Multiple myeloma	1
Mycosis fungoides	1
Total	100

Favorable clinical trials have been reported in Hodgkin's disease, other lymphomas, and chronic lymphocytic leukemia.[1, 3-8]

Since December 1954, CB 1348 has been provided to us through the courtesy of Alexander Haddow of the Chester Beatty Research Institute of the Royal Marsden Hospital in London, England and, later, through the courtesy of Donald S. Searle of Burroughs Wellcome & Co., Tuckahoe, N. Y.

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* The investigation on which this paper is based was supported by a grant from the Dorothy H. and Lewis Rosenstiel Foundation, New York, N. Y.

† Chlorambucil or Leukeran (Burroughs Wellcome & Co., Tuckahoe, N. Y.).

In our first publication[1] we presented the results in 42 patients, with special emphasis on Hodgkin's disease. Our clinical experiences with CB 1348 have now been extended to 100 patients: 47 with Hodgkin's disease, 18 with chronic lymphocytic leukemia, 16 with monocytic leukemia, 8 with lymphosarcoma, 5 with reticulum cell sarcoma, 3 with lymphocytic lymphoma without leukemia (Cohnheim), 1 with acute lymphocytic leukemia, 1 with multiple myeloma, and 1 with mycosis fungoides (TABLE 1).

Drug Administration and Dosage

CB 1348 was administered by mouth, in either 2- or 5-mg. tablets, given preferably before meals, in divided doses two or three times daily.

In patients with Hodgkin's disease, reticulum cell sarcoma, and monocytic leukemia the initial therapeutic dosage in most patients was 0.3 mg. per kg. per day for 21 to 28 days. In patients with chronic lymphocytic leukemia, lymphosarcoma, and lymphocytic lymphoma without leukemia (Cohnheim) the initial therapeutic dosage was, in most cases, 0.15 to 0.2 mg. per kg. per day for 21 days.

In our earlier clinical trials we discontinued all medication after each course of therapy. Eighteen patients who relapsed received a second course of therapy, 3 patients received a third course, and 1 patient received a fourth course. Later, however, most patients were continued after the first 21-day course on the following maintenance doses of CB 1348 in an attempt to prevent relapses: 2 to 6 mg. daily in patients with Hodgkin's disease and reticulum cell sarcoma, and 2 to 5 mg. two or three times weekly in patients with chronic lymphocytic leukemia, lymphosarcoma, and lymphocytic lymphoma without leukemia.

Toxic Effects

Most patients tolerated the medication without showing gastrointestinal side effects. Only a few patients complained of nausea and anorexia during the course of therapy, but these symptoms were mild and in no way interfered with the patients' activities. However, in 2 of our 100 patients, both with Hodgkin's disease, it was necessary to discontinue the CB 1348 therapy because of intractable nausea and vomiting. In one of these patients the symptoms were not manifested until the second course of therapy.

The majority of our patients developed slight to moderate leukopenia, anemia, and thrombocytopenia. The maximum depression was not usually observed until approximately 6 weeks after treatment was initiated. In most cases the depressant effect on the bone marrow was moderate and rapidly reversible, even in patients who already had a hypoplastic marrow as a result of other previous treatment. However, in 5 of our 100 cases, a marked hypoplasia of the marrow developed after CB 1348 therapy, although in all of these instances, on discontinuance of the drug, the depressant effect on the bone marrow was rapidly reversible, and no deaths attributable to the drug were observed.

Results

Hodgkin's disease. A total of 47 patients with Hodgkin's disease, 26 males and 21 females, was treated. Their age distribution was as follows: 19

patients were between 10 and 30 years, 20 were between 30 and 50 years, and 8 were over 50 years of age. The duration of illness before CB 1348 was initiated was from 3 months to 14 years. The diagnosis in all cases was established by lymph node biopsy.

With the exception of 4 patients, all had received previous treatment: 16 had received only X-ray therapy, from 1 to 7 courses; 14 had received both deep X-ray therapy and intravenous nitrogen mustard; 5 had received X-ray therapy and TEM; 4 had received X-ray therapy, nitrogen mustard, and TEM; and 4 had received nitrogen mustard alone. Four patients had undergone surgery for complications related to Hodgkin's disease: 3 of these had had a splenectomy and 1 had had a laminectomy with removal of an epidural Hodgkin's mass. All patients treated with CB 1348 manifested symptoms of generalized disease. Most of them were in the advanced stages of the disease and had previously failed to respond to X-ray therapy and/or nitrogen mustard and/or TEM.

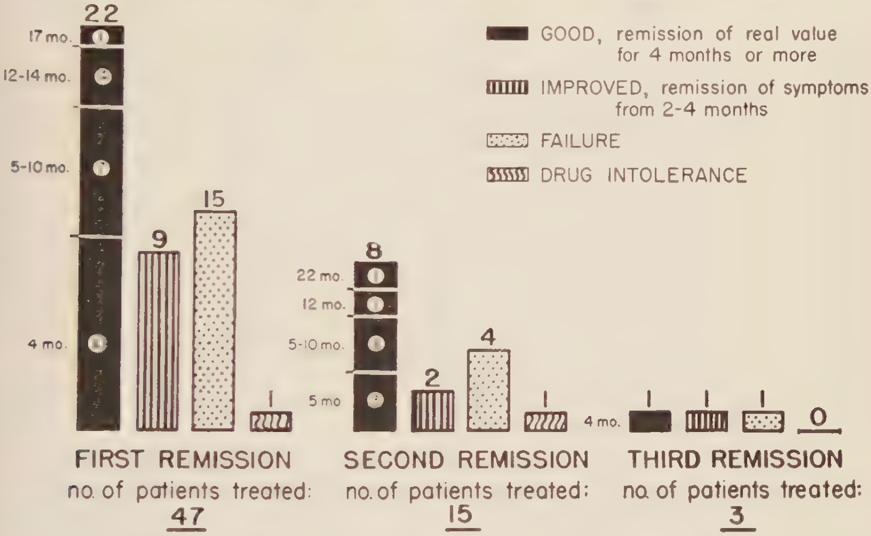

FIGURE 2. Results of treatment with CB 1348 in Hodgkin's disease.

The results of treatment with CB 1348 in Hodgkin's disease are summarized in FIGURE 2. Among 47 patients who received the first course of therapy, 15 failed to respond, and in 1 patient the drug was discontinued because of marked nausea.

Nine patients were "improved," meaning that the drug induced a remission of their symptoms, of real value but of shorter duration, lasting from 2 to 4 months. Three of these patients are still in their first remission; five have received a second course of CB 1348, 1 had a second complete "good" remission that is still in progress after 22 months; 2 improved; 2 failed to respond; and in 1 patient the therapy was changed.

Twenty-two of these patients with Hodgkin's disease had a "good" remission, meaning that the drug induced an improvement of real value to the patient so that, as a result of this therapy, something approaching a return to normal life was achieved for a period of 4 months or longer. The duration of the remission in this group of patients was as follows: in 10 patients the remission lasted for 4 months, in 7 patients 5 to 10 months, in 4 patients 12 to 14 months, and 1 patient is still in a continuing remission after 17 months.

Seven of these patients are still in their first remission. Nine of them received a second course of therapy: 7 obtained a second good remission (6 of these 7 patients are still in their second remission) and 2 showed improvement. One patient could not complete a second course of therapy because of intolerance to the drug, as expressed by intractable nausea and vomiting. The other 5 patients either failed to respond to a second course of therapy or their therapy was changed because it was felt that no further benefit could be obtained with CB 1348.

Three of the patients who responded to a second course received a third course of therapy: 1 obtained a good remission, 1 improved, and 1 failed to respond. A fourth course of therapy was given to the 1 patient who had showed a third good remission, and he had improvement of symptoms but of shorter duration. For this reason therapy was changed (FIGURE 4).

The favorable effects noted in the patients who responded to CB 1348 were as follows: there was a rapid return of the fever to normal, usually during the third to twelfth day of therapy; lymphadenopathy, splenomegaly, and hepatomegaly decreased during therapy, but the organs achieved their normal size approximately 6 weeks after the therapy was initiated; a progressive gain in weight was observed; jaundice, hepatomegaly, and liver functions were improved in 2 patients who presented such symptoms and signs before therapy was initiated.

It is of interest to note that one of our patients with Hodgkin's disease, a 25-year-old white female, received CB 1348 therapy of 16 mg. daily for 21 days during the early part of her pregnancy with no apparent deleterious effect on mother or fetus.

A summary of the case histories and a graphic representation of the course of the illness in two selected patients with Hodgkin's disease who reflected a good remission from CB 1348 treatment are presented herewith:

Case 1. M. K. This 41-year-old white woman had a diagnosis of Hodgkin's disease made by biopsy of a cervical lymph node in 1953. She was treated with three courses of deep X-ray therapy over abdominal, right cervical, and supraclavicular lymph nodes and spleen, intravenous nitrogen mustard, and three courses of oral TEM, cortisone, and phenylbutazone. She became refractory to all of this therapy. In January 1954 she again developed fever, anorexia, marked generalized lymphadenopathy, and marked splenomegaly. Her weight was 119 lb. Her peripheral blood cell count showed red blood cells to be 1,590,000 per cu. mm.; white blood cells, 3800 per cu. mm.; platelets, 78,660 per cu. mm.; and hemoglobin, 6.3 gm. per 100 cc. She was transfused before therapy was started and was then given CB 1348, 20 mg. daily (0.4 mg. kg.) for 30 days. Her fever abated after 3 days on therapy. The enlarged lymph nodes and spleen

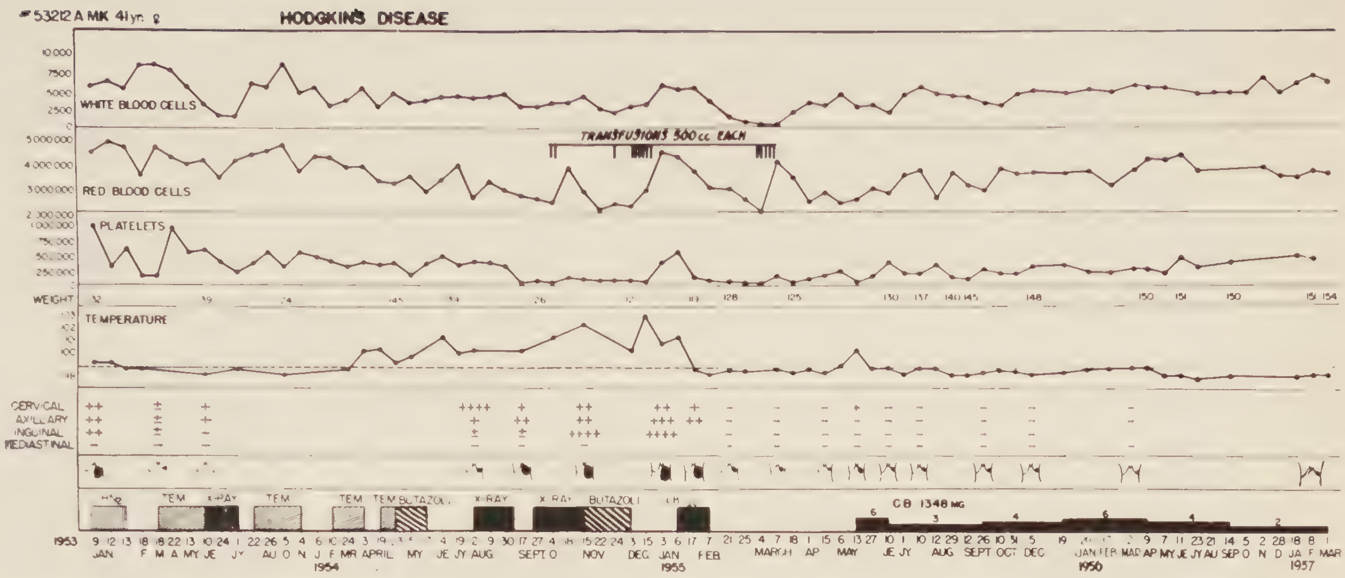

FIGURE 3. *Case 1*. Hodgkin's disease of 3 years' duration, in an advanced stage and refractory to conventional therapy. Two good remissions were obtained with CB 1348 treatment. The second remission has been in progress for 22 months.

regressed, and she gained 41 lb. in weight. The red blood cell level rose and remained about 3,500,000 per cu. mm.; hemoglobin rose to 11.8 gm. per 100 cc. The remission lasted for 5 months, after which she had a rise in temperature to 100° F., recurrent lymphadenopathy, and splenomegaly. She was then given 6 mg. of CB 1348 daily for 21 days and was placed immediately on a maintenance dosage of 3 to 4 mg. daily for the next 15 months. For the past 6 months she has been maintained on 2 mg. per day. This second complete remission therefore has been in progress for 22 months (FIGURE 3) and shows no signs of relapse to the present time.

Case 2. E. N. A 31-year-old white man had a diagnosis of Hodgkin's disease made by biopsy of a cervical lymph node in November 1954. In March 1955, he developed fever, anorexia, marked generalized lymphadenopathy, splenomegaly, and marked pruritus. Laboratory results included red blood cells, 4,390,000 per cu. mm.; white blood cells, 12,100 per cu. mm.; platelets, 1,097,500 per cu. mm.; hemoglobin, 12.7 gm. per 100 cc. His weight was 130 lb. (58.5 kg.). The patient was given CB 1348 in a dosage of 20 mg. daily (0.3 mg./kg.) for 21 days. Within the first 14 days of receiving the drug, fever and sweating abated and the pruritus was relieved. Rapid regression in the size of the enlarged cervical, axillary, and inguinal nodes and spleen followed. The peripheral blood remained essentially unchanged. After 2 months in remission the patient had gained 7 lb. At this time he had a recurrence of fever (101° F.), moderate lymphadenopathy, splenomegaly, and marked pruritus. He was then given a second course of CB 1348 in a dosage of 20 mg. daily for 21 days, followed by 3 to 4 mg. daily as a maintenance dosage. A second complete remission, lasting 7 months, was obtained. The patient relapsed again and received a third course of therapy with good results. A fourth remission was obtained, but this was of shorter duration. Subsequently this patient was treated with X-ray therapy to both axillary regions, plus nitrogen mustard. Currently he has been in his fifth remission for 2 months (FIGURE 4).

Chronic lymphocytic leukemia. A total of 18 patients with chronic lymphocytic leukemia, 13 males and 5 females, has been treated. Their age distribution was as follows: 2 patients were between 40 and 50 years, 6 between 50 and 60 years, and 10 over 60 years of age. The lapse between the hematological diagnosis and the initiation of CB 1348 therapy was from 1 month to 12 years. The diagnosis in all patients was confirmed by peripheral blood and bone marrow examinations. Four patients were treated initially with CB 1348 alone, the other 14 had previously received P^{32} or X-ray therapy. Previous to therapy, two patients had had a splenectomy for secondary hemolytic anemia. Fifteen patients showed splenomegaly and lymphadenopathy, and one of the splenectomized patients showed lymphadenopathy.

Ten of the 18 patients were anemic before therapy was initiated: in 3 the anemia was due to replacement of the normal elements of the marrow by lymphocytes (myelophthisis); in 3 it was due to myelophthisis plus depression of the normal elements by previous therapy; and in 4 it was due to a combination of myelophthisis plus secondary hemolytic anemia. All

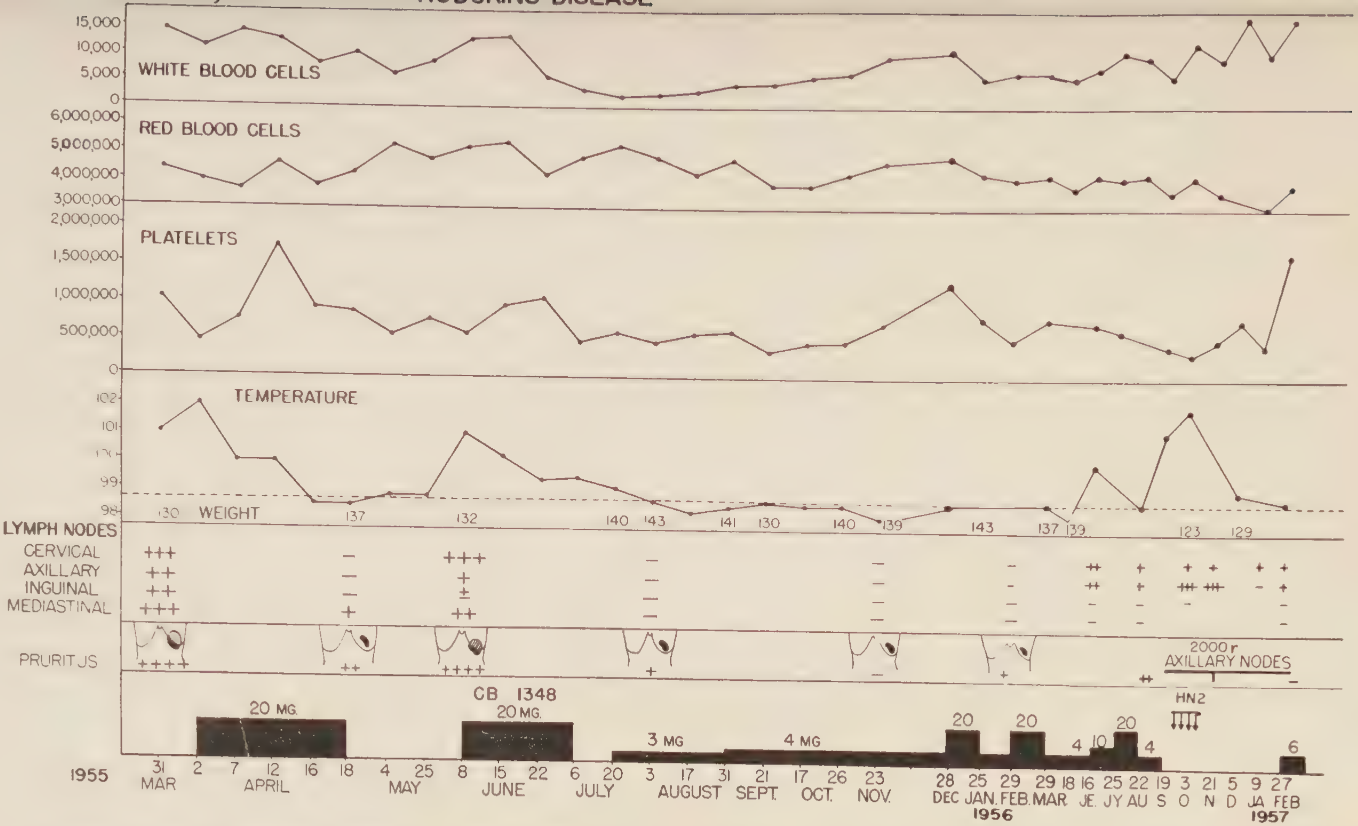

FIGURE 4. *Case 2.* Hodgkin's disease of 1 year's duration, with generalized symptoms and marked pruritus. Four remissions were obtained with CB 1348 treatment. In view of the shorter duration of the last remission, therapy was changed to a combination of nitrogen mustard and local X-ray therapy. A fifth remission was obtained and has been in progress for 3 months.

patients but one, after the initial therapeutic dose, were kept on a maintenance dose of from 2 to 4 mg. 2 to 3 times weekly.

The results of CB 1348 therapy in chronic lymphocytic leukemia are summarized in FIGURE 5. Twelve patients obtained a good remission, both clinical and hematological: the lymphadenopathy and splenomegaly subsided, the white blood cell count fell to normal limits, and the polymorphonuclear leukocyte count increased. In 2 of these patients the remission has been in progress for 11 months; in 3, for 5 to 7 months; and in 7, for 4 months. Five of these 12 patients in whom a remission was obtained with CB 1348 had previously failed to respond to P^{32} or to X-ray therapy.

Six patients failed to respond to CB 1348 therapy. Only one of these patients had CB 1348 as the initial therapy; she was a 54-year-old Negro female who had marked generalized lymphadenopathy and splenomegaly and showed practically complete replacement of the normal elements of the marrow by small lymphocytes. She received from 12 to 20 mg. daily for 2 months. The white blood cell count decreased from 225,000 to 160,000 per cu. mm., but no clinical or hematological improvement was otherwise obtained. The patient died from an intercurrent pneumonia. The other 5 patients who failed to respond to CB 1348 were in a very advanced stage of their disease, and they had already failed to respond to P^{32} and or X-ray therapy.

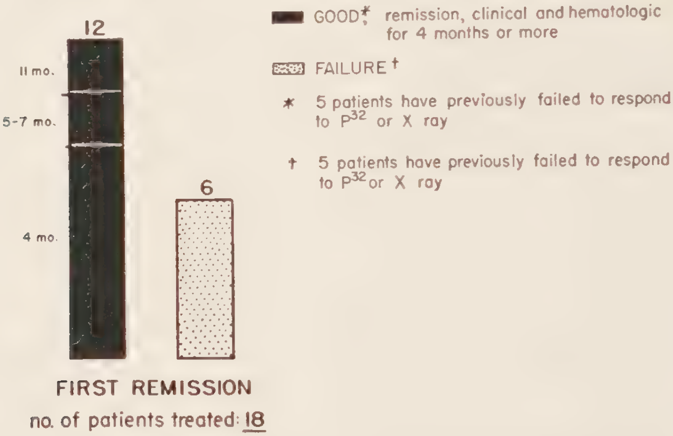

FIGURE 5. Results of treatment with CB 1348 in chronic lymphocytic leukemia.

It is of interest to emphasize that the patients who showed a hemolytic anemia as a complicating phenomenon when treated with CB 1348 obtained a clinical and hematological remission of their chronic lymphocytic leukemia, although the hemolytic anemia persisted. Additional therapy with prednisone was necessary in order to control the hemolytic anemia. This was demonstrated in Case 3 (FIGURE 6).

It is also important to note that the platelets were not significantly depressed in any of our patients while receiving CB 1348. On the contrary, those patients who were thrombocytopenic secondary to myelophthisis and who responded to CB 1348 therapy also showed a return to normal platelet levels (FIGURE 6).

The case histories with a graphic representation of the course of illness in two selected patients who obtained a remission from CB 1348 treatment are presented:

Case 3. M. T. A 50-year-old white woman first seen at University Hospital on April 17, 1956, with a 2-month history of gradual onset of fatigue, intermittent swelling of the legs, generalized lymphadenopathy, and abdominal distention. The physical examination revealed large and numerous cervical, axillary, inguinal, and abdominal nodes, the latter varying from 4 to 10 cm. in size. The spleen was palpable 5 cm. below the left costal margin, the liver 3 cm. below the right costal margin. Examination of the

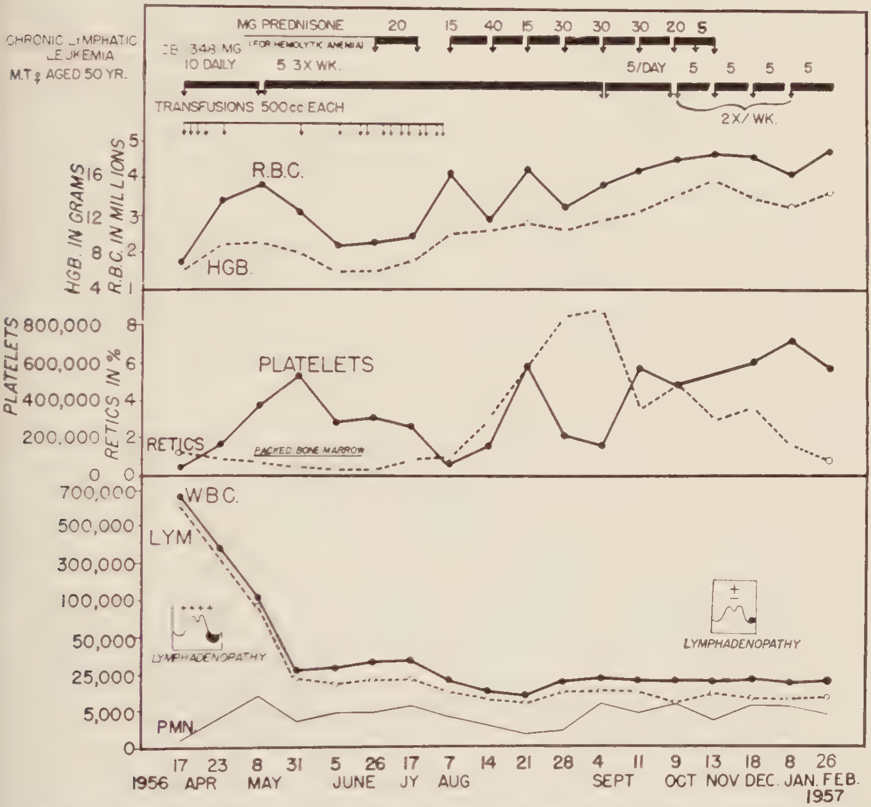

FIGURE 6. *Case 3.* Chronic lymphocytic leukemia with marked generalized lymphadenopathy, splenomegaly, thrombocytopenia, and complicated by secondary hemolytic anemia. There was complete replacement of the normal elements of bone marrow by lymphocytes. A remarkable remission was obtained with CB 1348. Hemolytic anemia did not subside until prednisone therapy was instituted. The patient was maintained on CB 1348 for 11 months and is still in a clinical and hematological remission.

legs revealed bilateral edema. Her peripheral blood cell count showed: red blood cells, 1,740,000 per cu. mm.; white blood cells, 689,000 per cu. mm., with 98 per cent lymphocytes and 2 per cent polymorphonuclear cells; platelets, 31,320; hemoglobin, 5.2 gm. per 100 cc; and reticulocytes, 3.4 per cent. Examination of the sternal bone marrow showed practically complete replacement of all normal elements by small mature lymphocytes. The direct Coombs test was negative; the indirect Coombs test was positive.[11]

The patient received 10 mg. of CB 1348 daily for 21 days, then 5 mg. daily for 7 days, followed by a maintenance dosage of 5 mg. 2 to 3 times weekly. The lymph nodes, spleen, and liver gradually diminished in size, and the total white blood cell count returned to normal levels. However, the hemolytic anemia persisted, and the patient required several blood transfusions.

On June 6, 1956 the patient was started on prednisone, following which the anemia subsided. Steroids were administered for 4 months and then discontinued.

This patient has been maintained on CB 1348 therapy for 11 months and is still in a clinical and hematological remission. Her last blood count on February 26, 1957, was: total red blood cell count, 4,700,000 per cu. mm.; total white blood cell count, 18,300, with 22 per cent polymorphonuclear cells, 61 per cent lymphocytes, and 1 per cent monocytes; platelets, 554,600 per cu. mm.; hemoglobin, 14.3 gm. per 100 cc; reticulocytes, 0.6 per cent (FIGURE 6).

Case 4. A. C. A 45-year-old white male, diagnosed in March 1956 as having chronic lymphocytic leukemia while hospitalized for respiratory symptoms. The physical examination revealed discrete cervical and axillary lymph nodes and a spleen the tip of which was palpable on deep inspiration. The patient's peripheral blood cell count was: red blood cells, 5,280,000 per cu. mm.; white blood cells, 67,000, with 16 per cent polymorphonuclear cells and 84 per cent lymphocytes; platelets, 612,480 per cu. mm.; hemoglobin, 15.9 gm. per 100 cc; reticulocytes, 1.4 per cent; and the hematocrit, 53 per cent. He was treated with CB 1348, 10 mg. daily for 34 days, then 5 mg. daily for 28 days, followed by 5 mg. two or three times weekly. The lymph nodes and spleen returned to normal size and the white blood cell count fell to 10,450 per cu. mm., with 38 per cent lymphocytes and 65 per cent polymorphonuclear cells. The red blood cells, hemoglobin, platelets, and reticulocytes remained unchanged.

The patient has been maintained on CB 1348 for 11 months and is still in a clinical and hematological remission (FIGURE 7).

Lymphosarcoma and lymphocytic lymphoma without leukemia (*Cohnheim*). A total of 11 patients, 6 males and 5 females, was treated. Eight of these patients had lymphosarcoma and 3 had lymphocytic lymphoma without leukemia. Their age distribution was as follows: 2 were between 20 and 30 years, 5 between 30 and 50 years, and 4 between 50 and 61 years of age The duration of illness before CB 1348 was initiated was from 3 months to 6 years. The diagnosis in all cases was confirmed by lymph node biopsy All patients manifested symptoms of generalized disease and most of them were in the advanced stages of the disease. All had received previous therapy consisting of X-ray, P^{32}, nitrogen mustard, and/or TEM. Nine of the 11 patients had failed to respond to previous therapy.

The results of CB 1348 therapy in lymphosarcoma are summarized in FIGURE 8. Of 8 patients treated, 6 obtained a good remission, both clinical and hematological; one of these is still in a remission after 9 months, the other 5 are still in remission after 4 months. One patient improved; this patient, a 53-year-old male, lived for 9 months, and the clinical and hematological symptoms did not completely subside, but it was felt that CB 1348 inhibited the rapid progression of the lymphosarcoma. One patient failed to respond to CB 1348 therapy.

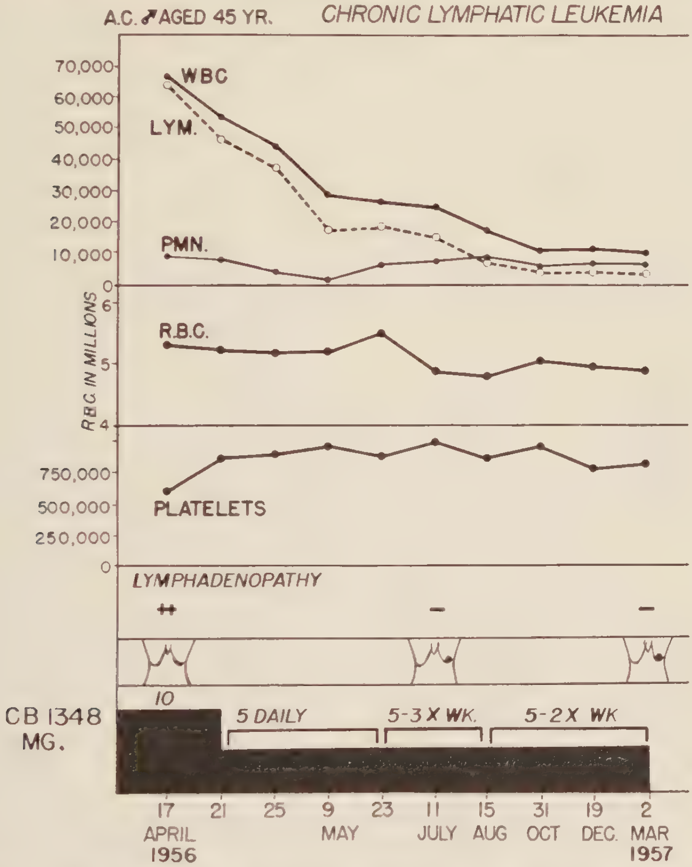

FIGURE 7. *Case 4.* Chronic lymphocytic leukemia with moderate lymphadenopathy and splenomegaly. A clinical and hematological remission was obtained with CB 1348 and has been in progress for 11 months.

It is of interest to remark that 2 of the patients with lymphosarcoma who obtained a good remission had subcutaneous infiltrative lesions with lymphosarcoma cells, and one had lymphosarcoma involving the skin of the face and trunk. In all 3 patients the lesions cleared on CB 1348 therapy, in spite of the fact that they had been unsuccessfully treated previously with TEM, X-ray therapy, and/or nitrogen mustard.

Of the 3 patients with lymphocytic lymphoma without leukemia, 2 obtained a remission; in 1 the remission has been in progress for 11 months, in the second for 4 months. The third patient has improved, and CB 1348 seems to have inhibited the rapid progression of the generalized disease. However, the hematological and clinical signs and symptoms have not completely subsided after 6 months of therapy.

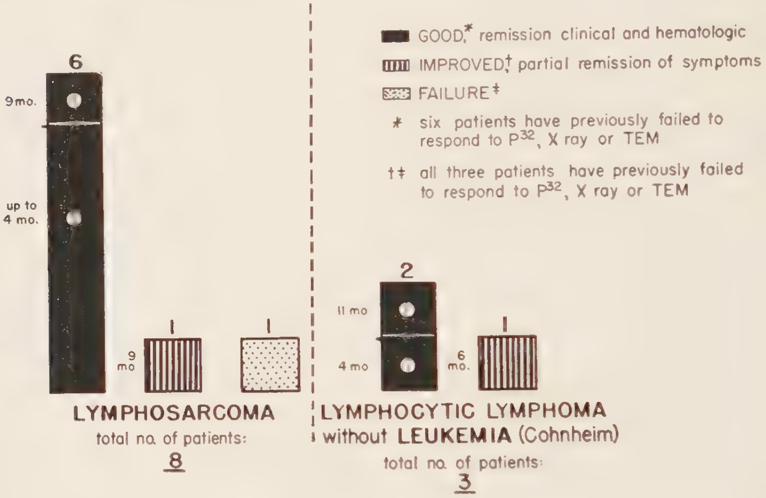

FIGURE 8. Results of treatment with CB 1348 in lymphosarcoma and lymphocytic lymphoma without leukemia (Cohnheim).

The case history and a graphic representation of the course of illness in one selected patient of this group with lymphosarcoma who obtained a remission from CB 1348 treatment are presented:

Case 5. G. L. A 26-year-old white male was first diagnosed as having lymphosarcoma with a lymph node biopsy in April 1954. He was treated with X-ray therapy, and his general health remained satisfactory, until November 1955, when he became aware of marked fatigue. At this time he had marked generalized lymphadenopathy and a spleen palpable 13 cm. below the left costal margin. His peripheral blood count was: total red blood cells, 2,580,000 per cu. mm.; total white cells, 4950 per cu. mm., with 38 per cent lymphocytes, 48 per cent polymorphonuclear cells, 2 per cent eosinophils, and 12 per cent monocytes; hemoglobin was 7.5 mg. per cent. From November 27, 1955 to February 9, 1956 he received 9 blood transfusions. On February 20, 1956 he was admitted to University Hospital where he received 2 more blood transfusions and X-ray therapy consisting of 1000 r over the spleen, mediastinal, and axillary nodes. No change in the size of the spleen or lymph nodes was noted on X-ray therapy, and the hemolytic anemia persisted. On March 30, 1956, the patient was readmitted for treatment of homologous serum jaundice. At this time he was placed on prednisone therapy and, on June 1, 1956, on CB 1348, 6 mg. daily for 21 days, followed by 5 mg. 2 to 3 times a week. On this combination of therapy the spleen and lymph nodes returned to normal in size, the patient regained his strength, his appetite improved, and he was able to return to his full-time employment as an electrician. His anemia subsided, and he has not required any transfusions since February 1956. This patient has lived a normal life and is still in a remission, both clinical and hematological, after 11 months (FIGURE 9).

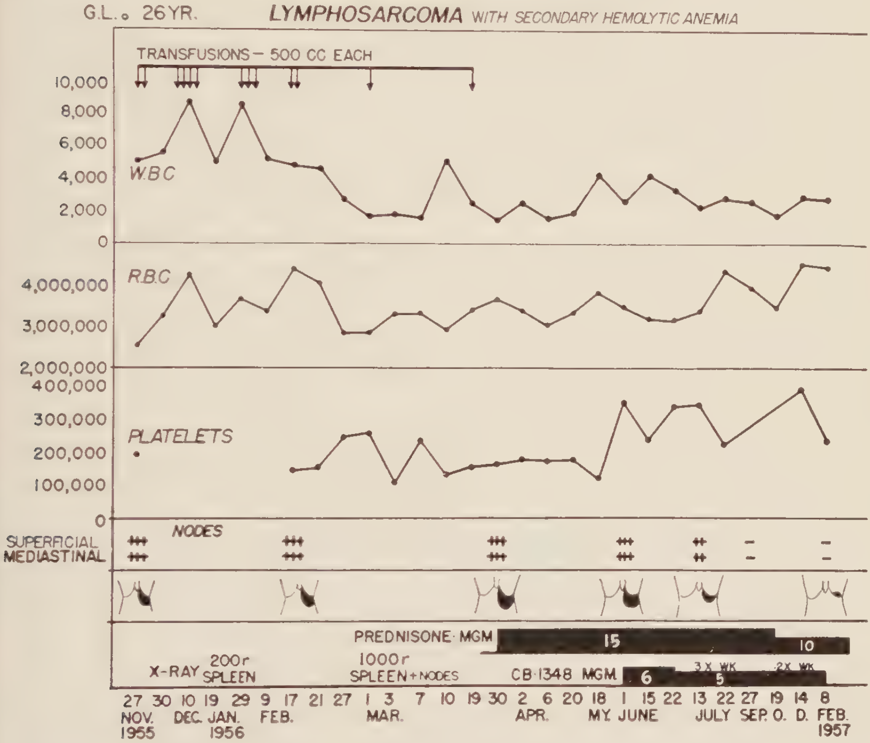

FIGURE 9. *Case 5.* Lymphosarcoma with generalized lymphadenopathy, marked splenomegaly, and complicated by secondary hemolytic anemia. The condition was refractory to X-ray therapy. A remarkable remission was obtained with CB 1348. Hemolytic anemia subsided on prednisone therapy.

Reticulum cell sarcoma. Five patients with reticulum cell sarcoma were treated with CB 1348; 3 were males and 2 females. One patient was 20 years old and the other 4 were over 50 years of age.

The results of therapy are presented in FIGURE 10. Only one patient had a good remission for 6 months; he was a 64-year-old white male with many palpable abdominal masses and large retroperitoneal and retrogastric tumors demonstrated by X ray. A marked regression in the abdominal masses was obtained after therapy. The patient felt good, gained 27 lb. in weight, and returned to his normal activities for 6 months. At this time he relapsed and was treated again with a second course of CB 1348, but failed to respond to this therapy, and a second remission was not obtained. One patient was temporarily improved for 1 month on CB 1348 therapy. The other 3 patients failed to respond.

Monocytic leukemia. Sixteen patients with monocytic leukemia, 10 females and 6 males, were treated with CB 1348. Their age distribution was as follows: 4 between 10 and 30 years, 4 between 30 and 50 years, and 8 over 50 years of age.

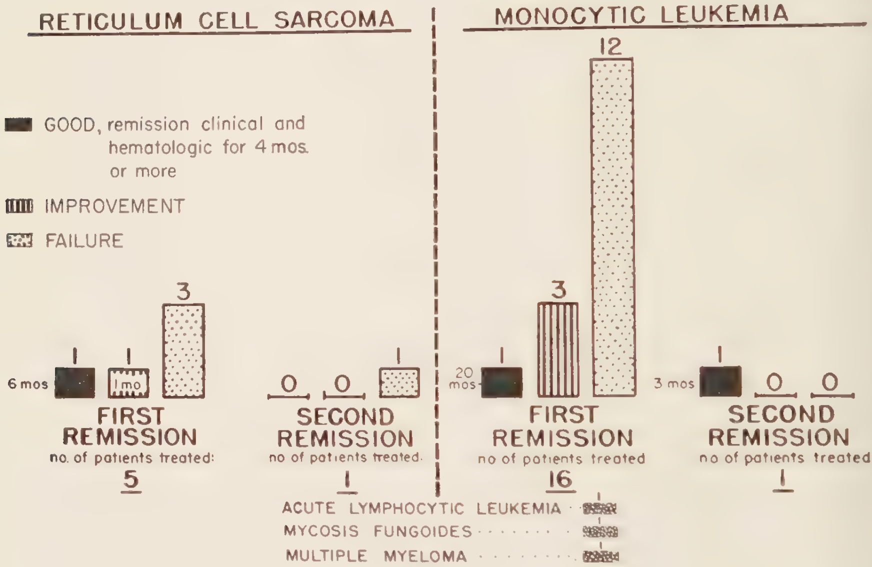

FIGURE 10. Results of treatment with CB 1348 in reticulum cell sarcoma and monocytic leukemia.

The results of therapy with CB 1348 are summarized in FIGURE 10. In general, the results have been disappointing: 12 patients failed to respond to an adequate trial of CB 1348, and 3 exhibited a temporary decrease of white blood cells without improvement in the red blood cells, platelets, percentage of monoblasts in the bone marrow, or any influence on the course of the disease. Only one patient, a 45-year-old white woman with monocytic leukemia, benefited from CB 1348 therapy. Laboratory findings on admission included red blood cells, 2,510,000 per cu. mm.; white blood cells, 1350 per cu. mm.; hemoglobin, 9.1 gm. per 100 cc; platelets, 90,000 per cu. mm.; reticulocytes, 5.2 per cent. The bone marrow showed almost complete replacement of normal elements by monoblasts. The patient received a course of CB 1348 in a dosage of 22 mg. daily (0.3 mg. kg.) for 14 days. A complete remission, both clinical and hematological, including normal peripheral blood and bone marrow, was obtained; it lasted for 20 months.

At this time the patient relapsed, her peripheral blood count was: total white blood cell count, 1050 per cu. mm.; total red blood cell count, 1,340,000 per cu. mm.; reticulocytes, 1.6 per cent; platelets, 17,400 per cu. mm.; and hemoglobin 4.9 gm. per 100 cc. A bone marrow aspiration showed replacement of all normal elements by monoblasts, with 92 per cent monoblasts counted in the marrow differential. The patient was treated with 20 mg. of CB 1348 daily for 3 weeks. A second complete clinical and hematological remission was obtained, and it has been maintained now for 3 months.

One patient with acute lymphocytic leukemia, one with multiple myeloma, and one with mycosis fungoides did not obtain any improvement when treated with CB 1348.

Comments

CB 1348 has proved to be of value in the therapy of Hodgkin's disease, chronic lymphocytic leukemia, lymphocytic lymphoma without leukemia (Cohnheim), and lymphosarcoma.

Twenty-two of 47 patients with Hodgkin's disease obtained a remission after the first course of therapy. In 12 of these patients the remission lasted for 5 months or more; the longest remission thus far has continued for 17 months. Among 15 patients who received a second course of therapy, 8 obtained a second remission; in 5 of these patients the remission lasted 5 months or more, the longest remission being 22 months in duration. Three patients received a third course of therapy, and only 1 obtained a remission. Some of the patients who obtained a remission on CB 1348 were in an advanced stage of their disease and had not responded to conventional therapy. Although remission in some patients was of short duration, a second remission was obtained in most patients treated, and a third remission was obtained in 1 of 3 patients treated.

In our hands CB 1348 has been safer than TEM because it does not have the erratic and sometimes unpredictable marrow-depressant effect that characterizes TEM.[9-12] In some cases CB 1348 is preferred to nitrogen mustard because it can be given orally, is usually free of side effects, and does not depress the peripheral blood and bone marrow as much as nitrogen mustard.[13-15]

It is difficult to compare CB 1348 with X-ray therapy in the treatment of Hodgkin's disease, since neither sufficient patients with localized disease nor patients in the initial stages of the disease have been treated with CB 1348. However, it is felt that local irradiation should first be instituted in the presence of proved localized disease. This treatment may be supplemented or replaced by CB 1348 in the presence of manifestations of generalized spread of the disease, in cases of poor general condition (yet in which there are no demonstrable active lesions), and in cases resistant to X-ray therapy.

Twelve of 18 patients with chronic lymphocytic leukemia obtained a remission on CB 1348 therapy. In 5 of these patients the remission has lasted 5 months or more; the longest remission thus far recorded and still continuing is 11 months. Five of these 12 patients had previously become resistant to P^{32} or X-ray therapy. The hemolytic anemia complicating some of the chronic lymphocytic leukemias was not corrected by CB 1348 therapy alone. With the addition of prednisone to the CB 1348 therapy the hemolytic anemia was controlled. CB 1348 was effective in some patients with a myelophthisic marrow in whom anemia and thrombocytopenia were predominant. It is significant that the platelets were not further depressed by CB 1348 therapy in any of our patients; on the contrary, in those in whom the thrombocytopenia was due to the myelophthisis, the platelets increased to normal levels as part of the response to therapy.

It is too early to compare the effect of CB 1348 to P^{32} or X ray in patients with chronic lymphocytic leukemia, since our longest remissions are now only 11 months, as compared to those of some patients followed as long as 13 years after treatment with P^{32} or X ray.[16] However, it is important to emphasize that some patients, after becoming resistant to P^{32}, have obtained a remission on CB 1348.

Six of 8 patients with lymphosarcoma responded to CB 1348 therapy; 2 of these had previously failed to respond to P^{32}, X ray, and/or TEM. Three of these patients had subcutaneous or cutaneous infiltrations with lymphosarcoma cells that cleared after treatment. Two of 3 patients with lymphocytic lymphoma without leukemia responded to CB 1348. It is difficult to compare the effect of CB 1348 in the treatment of the above 2 lymphomas to X ray, P^{32}, or TEM. However, it is important to note that some patients with generalized spread of the disease who have failed to respond to conventional therapy have shown a satisfactory response to CB 1348.

Only 1 of 5 patients with reticulum cell sarcoma and 1 of 16 patients with monocytic leukemia obtained remissions on CB 1348 therapy.

Summary

To date, 100 patients have been treated in our Hematology Clinic with *p*-bis(2-chloroethyl)aminophenylbutyric acid (CB 1348), of whom 59 showed a more or less favorable response. These include: 47 patients with Hodgkin's disease, 18 with chronic lymphocytic leukemia, 16 with monocytic leukemia, 8 with lymphosarcoma, 5 with reticulum cell sarcoma, 3 with lymphocytic lymphoma without leukemia (Cohnheim), and 1 each with acute lymphocytic leukemia, multiple myeloma, and mycosis fungoides.

"Satisfactory" responses were obtained in 22 patients with Hodgkin's disease, in 12 with chronic lymphocytic leukemia, in 6 with lymphosarcoma, in 2 with lymphocytic lymphoma without leukemia, in 1 with reticulum cell sarcoma, and in 1 with monocytic leukemia.

CB 1348 was practically free of side effects and it proved to be less damaging to the hematopoietic system than any other cytotoxic agent used thus far

References

1. Haddow, A. 1954. Experimental and clinical aspects of the action of various carboxylic acid derivatives in the aromatic nitrogen mustard series. *In* Ciba Foundation Symposium on Leukemia Research. :196. G. E. W. Wolstenholme & M. P Cameron, Eds. Little, Brown. Boston, Mass.

2. Bollag, W. 1953. Experimental investigations of the effect of cytostatic substances in the blood picture. Schweiz. med. Wochschr. **83**: 1027.
3. Galton, D. A. G., L. S. Israels, J. D. N. Navarro & M. Till. 1955. Clinical trials of *p*-(di 2-chloroethylamino) phenylbutyric acid (CB 1348) in malignant lymphoma. Brit. Med. J. **2**: 1172.
4. Bouroncle, B. A., C. A. Doan, B. K. Wiseman & W. J. Frajola. 1956. Evaluation of CB 1348 in Hodgkin's disease and allied disorders. A.M.A. Arch. Internal Med. **97**: 703.
5. Altman, S. J., A. Haut, G. E. Cartwright & M. M. Wintrobe. 1956. Early experience with *p*-(N_1N-di 2-chloroethyl) aminophenylbutyric acid (CB 1348), a new chemotherapeutic agent effective in the treatment of chronic lymphocytic leukemia. Cancer. **9**: 512.
6. Ultmann, J. E., G. A. Hyman & A. Gellhorn. 1956. Chlorambucil in treatment of chronic lymphocytic leukemia and certain lymphomas J. Am. Med. Assoc. **162**: 178.
7. Shanbrom, E., A. G. Knudson & S. Rappaport. 1957. Clinical evaluation of chlorambucil. (Abstr.) Clin. Research Proc. **5**: 31.
8. Hall, B. E., F. M. Willet, T. V. Feichtmeir, D. R. Hales, R. W. Jerner & J. Franco. 1957. Clinical evaluation of chlorambucil in the treatment of human neoplastic disease. (Abstr.) Clin. Research Proc. **5**: 83.
9. Paterson, E. & P. B. Kunkler. 1953. Triethylene melamine in human malignant disease. Brit. Med. J. **1**: 59.
10. Hamermon, D. J. & S. Malamed. 1953. Some clinical experience in the use of triethylene melamine (TEM). J. Mt. Sinai Hosp. N. Y. **20**: 16.
11. Burtner, C. W., L. C. Jensen & J. M. Rumball. 1955. Triethylene melamine in the treatment of lymphomas and other neoplastic diseases. Ann. Internal Med. **38**: 1272.
12. Slipyan, A. 1953. Unusual toxicity to triethylene melamine in a case of chronic myelogenous leukemia. Ann. Internal Med. **38**: 331.
13. Jacobson, L. O., C. L. Spurr, E. S. G. Barron, T. Smith, C. Lushbaugh & G. F. Dick. 1946. Studies on the effect of methyl-bis (β-chloroethyl) amine hydrochloride on neoplastic diseases and allied disorders of the hemopoietic system. J. Am. Med. Assoc. **132**: 263.
14. Zanes, R. P., C. A. Doan & H. A. Hoster. 1948. Studies in Hodgkin's syndrome. VII. Nitrogen mustard therapy. J. Lab. Clin. Med. **33**: 1002.
15. Goodman, L. S., M. M. Wintrobe, W. Dameshek, M. J. Goodman, A. Gilman, & M. T. McLennan. 1946. Nitrogen mustard therapy: use of methyl-bis (β-chloroethyl) amine hydrochloride for Hodgkin's disease, lymphosarcoma, leukemia and certain allied and miscellaneous disorders. J. Am. Med. Assoc. **132**: 126.
16. Doan, C. A., B. K. Wiseman, C. S. Wright, J. H. Geyer, W. Myers & J. W. Myers. 1947. Radioactive phosphorus P^{32}: a six year clinical evaluation of internal radiation therapy. J. Lab. Clin. Med. **32**: 943.

MYLERAN AND TRIETHYLENE MELAMINE IN THE TREATMENT OF CHRONIC GRANULOCYTIC LEUKEMIA

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Following the demonstration of the human antitumor activity of triethylene melamine (TEM) in 1950 by Rhoads and others,[1] this drug was extensively employed in the treatment of leukemia and lymphoma. From these clinical trials there emerged the general impression that, with respect to leukemia, TEM was most useful in the chronic lymphocytic form of the disease, in spite of adequate documentation of its effectiveness in chronic granulocytic leukemia.[2] The structural similarity of nitrogen mustard (HN2) and TEM as polyfunctional alkylating agents undoubtedly was instrumental in suggesting the major indications for the use and mode of therapeutic application of the latter drug; namely, emphasis on lymphoid rather than myeloid neoplasms, and the preference for intermittent courses of administration rather than continued therapy.

When 1,4-dimethanesulfonoxy-1,4-dimethylbutane (Myleran) was reported to be effective in chronic granulocytic leukemia,[3] the fact that its chemical structure differed radically from that of the ethylenimines seemed to establish a qualitative basis for the supposed preferential action of Myleran on myeloid, and of HN2 and TEM on lymphoid proliferative disease.

At the Simpson Memorial Institute, 41 patients with chronic granulocytic leukemia were treated with TEM from 1951 through 1956, and 31 patients were treated with Myleran between 1953 and 1956. Some of these patients had received other medication, such as radiation, urethan, or Colcemide, prior to being placed on TEM or Myleran. Some were in a relatively early well-differentiated stage of their disease, whereas in others the disorder was advanced and many of the granulocytes in the peripheral blood were immature. In this category were placed far-advanced cases with predominance of myeloblasts and atypical progranulocytes in the blood and marrow, as well as cases of moderately advanced disease, since the distinction between such degrees of severity is arbitrary and often varies among different clinicians. On the other hand, there is rarely disagreement over classifying a case in the early well-differentiated stage. The patients were classified in terms of the quality and duration of their therapeutic responses according to the following criteria. Excellent: leukocyte count less than 15,000 pe cu. mm. with early granulocytes (myeloblasts through metamyelocytes below 5 per cent; normal hemoglobin and packed volume of red cells; fre of symptoms referable to leukemia; spleen not palpable or barely palpable duration in this status 6 months or longer. Good: leukocyte count less than 50,000 with early granulocytes below 20 per cent; hemoglobin above 11 gm or packed volume of red cells above 30 per cent; virtually asymptomatic splenomegaly less than before treatment; duration in this status or bette

4 months or longer. Fair: leukocyte count and early granulocyte percentage reduced below pretreatment levels; hemoglobin and packed volume of red cells essentially stable; no serious incapacitation; duration in this status or better 2 months or longer. Poor: disease not under hematological or clinical control; serious incapacitation; indeterminate duration in this status.

Summaries of our therapeutic experience with triethylene melamine and Myleran are presented in TABLES 1 and 2. The TEM and Myleran groups are not comparable in every respect because the agents became available at different times and because there was a tendency to employ TEM, in the early period of its use, in cases becoming difficult to control with radiation or urethan. By contrast, Myleran was introduced as an effective form of therapy for chronic granulocytic leukemia and was used from the outset as initial treatment for many patients with this disease. Thus, of the 41 cases of chronic granulocytic leukemia treated with TEM, 10 were classified as early and 31 as advanced, 9 had had no previous treatment, and 32 had received earlier therapy. Of the 31 cases treated with Myleran, 16 were described as early and 15 as advanced, 12 had had no prior antileukemia medication, and 19 had been previously treated. These differences between the 2 groups, coupled with the fact that some time elapsed after TEM was introduced before the importance of continuous therapy was appreciated, are probably sufficient to explain the apparently superior results obtained with Myleran as shown by 8 excellent and 18 good responses in contradistinction to the 2 excellent and 15 good responses obtained with TEM.

Experience with 72 cases of chronic granulocytic leukemia treated with Myleran or triethylene melamine for 1 to 5 years has led to the following conclusions and procedures:

(1) The generally preferred initial dose of Myleran is 6 mg. daily; of TEM 5 mg. every other day.

(2) Continued therapy is preferable to intermittent courses of treatment.

(3) The maintenance dose requirement must be determined for each patient. In the case of Myleran, the range for the majority is 2 mg. twice weekly to 2 mg. daily; for TEM it is 2.5 mg. every 2 weeks to 5 mg. weekly.

(4) The most valuable single index by which to regulate dosage is the total leukocyte count, obtained at 2-week intervals, although the differential count, hemoglobin, platelet count, and spleen size also are important.

(5) In general, after a normal leukocyte level has been attained and a maintenance regimen instituted, the dosage should be increased if the count rises above 15,000 per cu. mm. and continued unchanged if it is between 6000 and 15,000; the medication should be discontinued if the count falls below 6000.

(6) In cases of overdosage, it may be necessary to omit medication for many months because of persistent leukopenia.

(7) With the dosages employed, no differences have been demonstrated in therapeutic efficacy or in myeloid depressive effects between Myleran and TEM.

(8) In cases becoming refractory to either of these agents, it has not been possible to obtain significant benefit by substituting the other.

TABLE 1

CHRONIC GRANULOCYTIC LEUKEMIA TREATED WITH TRIETHYLENE MELAMINE

Therapy	Number of cases	Dosages	Therapeutic responses and status of disease*			
			Excellent	Good	Fair	Poor
Untreated before TEM Intermittent courses	7	5 mg. each of 2–10 doses	0	3 (all early)	2 (both advanced)	2 (both advanced)
Untreated before TEM Continuous administration	2	5 mg. monthly to 5 mg. weekly	1 (early)	1 (advanced)	0	0
Other treatment before TEM Intermittent courses	32	5 mg. each of 2–13 doses	0	9 (3 early) (6 advanced)	6 (2 early) (4 advanced)	17 (all advanced)
Other treatment before TEM Continuous administration	4†	5 mg. monthly to 5 mg. weekly	1 (early)	2 (1 early) (1 advanced)	0	1 (advanced)
Total	41		2	15	8	20

* These categories include 4 duplications, resulting from some patients receiving both intermittent and continuous therapy.

† Prior intermittent treatment.

Table 2
Chronic Granulocytic Leukemia Treated with Myleran

Therapy	Number of cases	Dosages	Therapeutic responses and status of disease*			
			Excellent	Good	Fair	Poor
Untreated before Myleran Intermittent courses	6	12—*6*—4 mg. 10—*15*—30 days	0	5 (all early)	0	1 (early)
Untreated before Myleran Continuous administration	6 + 4†	2 mg. 3 times weekly to 2 mg. daily	6 (all early)	1 (advanced)	2 (both advanced)	1 (advanced)
Other treatment before Myleran Intermittent courses	9	12—*6*—4 mg. 10—*15*—30 days	0	3 (2 early) (1 advanced)	1 (early)	5 (1 early) (4 advanced)
Other treatment before Myleran Continuous administration	10 + 6†	2 mg. alt. days to 2 mg. daily	2 (both early)	9 (6 early) (3 advanced)	1 (early)	4 (1 early) (3 advanced)
Total	31		8	18	4	11

* These categories include 10 duplications, resulting from some patients receiving both intermittent and continuous therapy.

† Prior intermittent treatment.

(9) Results of continued therapy with either of these agents are superior to those obtained by the use of irradiation administered at irregular intervals determined by the activity of the disease.

(10) These data do not permit a definitive comparison between chemotherapy and regularly spaced, individually titrated irradiation (Osgood[4]). The indications are that the former is at least equal to the latter method of treatment.

References

1. Rhoads, C. P., D. A. Karnofsky, J. H. Burchenal & L. F. Craver. 1950. Triethylene melamine in the treatment of Hodgkin's disease and allied neoplasms. Trans. Assoc. Am. Physicians. **63**: 136.
2. Bond, W. H., R. J. Rohn, R. W. Dyke & P. J. Fouts. 1953. Clinical use of triethylene melamine. Report of seventy five cases. A.M.A. Arch. Internal Med. **91**: 602.
3. Haddow, A. & G. M. Timmis. 1953. Myleran in chronic myeloid leukaemia. Lancet. **1**: 207.
4. Osgood, E. E. 1951. Titrated, regularly spaced radioactive phosphorus or spray roentgen therapy of leukemias. A.M.A. Arch. Internal Med. **87**: 329.

THERAPY OF THE MYELOPROLIFERATIVE DISORDERS WITH MYLERAN*

By William Dameshek, Norma B. Granville,† and Fernando Rubio, Jr.
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The myeloproliferative disorders may be classified as proliferations of one or several of the cellular constituents of the bone marrow, of unknown etiology, and having a variable course and a highly variable clinical and hematological picture. The bone marrow of the adult is composed of red, actively growing cells, usually in the flat bones, and of yellow, potentially active material, usually in the long bones. This inactive marrow may become active under certain conditions such as chronic hemorrhage or continued active hemolysis. In addition, there are areas in the body, notably in the spleen and liver, the sites of previous embryonic blood formation, that have the potentiality of becoming foci of marrow growth; that is, myeloid metaplasia. Most of the proliferative function of the marrow is in response to well-defined stimuli such as infections or hemorrhage. Here the response is self-limited; it subsides as the stimulatory principle abates. In some states, however, the reason for the proliferation is quite obscure and, once the proliferative process has begun, it continues as a self-perpetuating disorder. It is probable that the latter conditions should be grouped with the neoplastic or leukemic proliferations.

Total, selective, and intermediate forms of myeloproliferative disease may be discriminated. Examples of total or near-total proliferation are polycythemia vera and myelofibrosis with myeloid metaplasia. Polycythemia vera is a very chronic, almost benign panmyelopathy, in which there is proliferation, not only of the red cell series, but of the granulocytes and megakaryocytes as well. Myelofibrosis with myeloid metaplasia, whether "agnogenic" or secondary to polycythemia vera, represents a proliferation of the reticulum of the bone marrow with eventual myelofibrosis, with simultaneously active proliferation of potential bone marrow in spleen, liver, and possibly long bones. Less widespread proliferations of the marrow, although more malignant in their nature, are cases of chronic granulocytic leukemia in which chiefly the granulocytes are proliferating, and acute granulocytic eukemia. Thrombocythemia is another example of "selective" marrow proliferation, limited largely to the megakaryocytes. It should be emphasized that borderline and transitional states are seen quite commonly, making exact classification in many cases quite difficult.

Chronic and acute forms of myeloproliferative disease may be discriminated (TABLE 1). It is possible that the Di Guglielmo syndrome represents an acute, highly malignant form of polycythemia vera, with proliferation of

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TABLE 1

A CLASSIFICATION BASED UPON THE DEGREE OF PROLIFERATION OF THE VARIOUS BONE MARROW ELEMENTS, AND UPON THE RAPIDITY OF THE GROWTH PROCESS*

	RBC	WBC	Megas.	R-E	Potential bone marrow metaplasia
Chronic					
Polycythemia vera	++	+	++	±	±
Chronic granulocytic leukemia	±	+++	+	±	±
Myelofibrosis	—	+	++	++	++
Myeloid metaplasia					
Thrombocythemia	±	±	+++	+	+
Acute					
Acute granulocytic leukemia	±	++	—	—	—
Di Guglielmo syndrome	++	+(++)	±	+	±

* With so many cellular elements participating, it is well to remember that "transitional" and "mixed" forms that are difficult to classify often occur.

Abbreviations: RBC, marrow nucleated red cells; WBC, marrow granulocytes; Megas. megakaryocytes; R-E, reticuloendothelial cells.

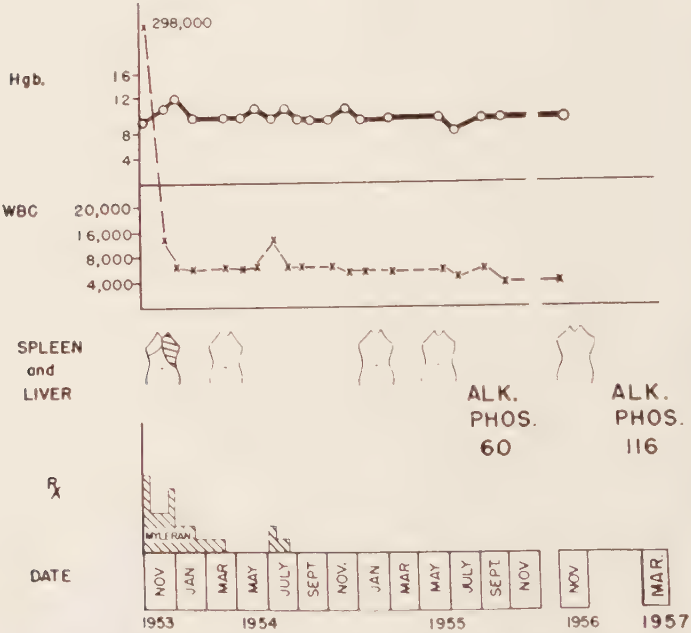

FIGURE 1. *Case L. F.* Chronic granulocytic leukemia, initial leukocyte count 298,0 Sustained complete clinical, hematological, and bone marrow remission induced by Myler Development of high normal or slightly elevated alkaline phosphatase levels of the mat granulocytes two to four years after remission was induced.

red cell, granulocyte, and possibly megakaryocytic tissue taking place. The varying pictures seen in the Di Guglielmo syndrome are probably due to the varying degree of proliferation of either the red cell or white cell elements; and this, in turn, depends upon the stage of the disorder. This condition probably begins first as a red cell proliferation, then becomes "mixed" with myeloblastic growth, eventually terminating as a pure myeloblastic condition.

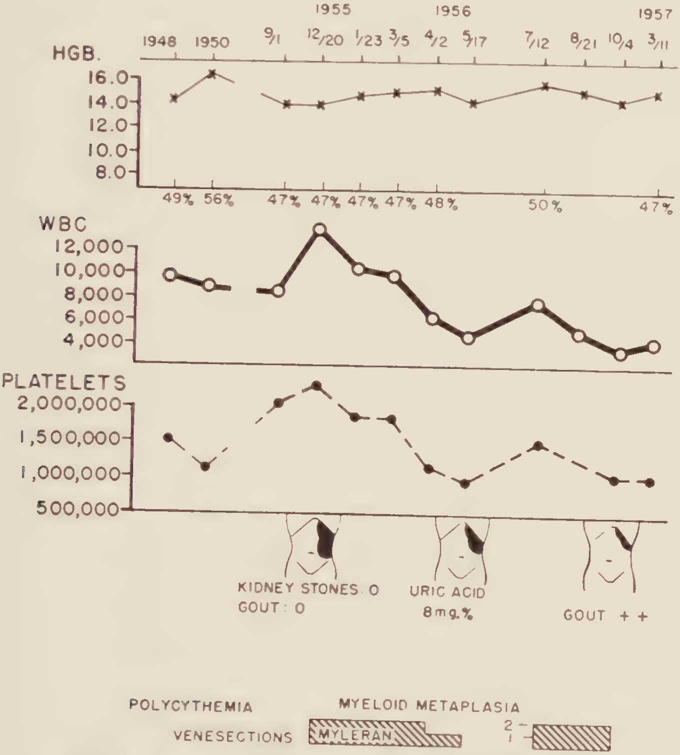

FIGURE 2. *Case N. W.* Polycythemia vera with transition to myelosclerosis myeloid netaplasia. Treatment with very small doses of Myleran. Reduction of spleen size, eukocyte, and platelet counts. Little if any effect on the erythrocyte count.

Therapy of the myeloproliferative disorders has been greatly advanced in ecent years with knowledge of the striking effects of 1,4-dimethanesulfonoxy-outane (Myleran) in chronic granulocytic leukemia. This alkylating agent ippears to have a highly selective, if not specific, effect upon bone marrow ells, notably of the granulocytic variety. In an experience of over 50 con-ecutive cases of chronic granulocytic leukemia, Myleran was found to induce triking and complete clinical, hematological, and marrow remissions. Its onsistency and predictability of action, whether due to the drug or to the lisease, is indeed remarkable. Within a month after a dosage of 6 to 16 mg. laily, the white count is almost at normal values and the spleen barely alpable. Within two months, complete normality is usually present. If, by dint of careful "titration" of the dose, the white count is allowed to drop to levels of approximately 5000 per cu. mm., lengthy and complete remissions may persist for several months to four years without further additional therapy. In three cases, the completeness of the remission was associated with a return of the alkaline phosphatase value of the mature granulocytes to high normal values from the previously very low values characteristic

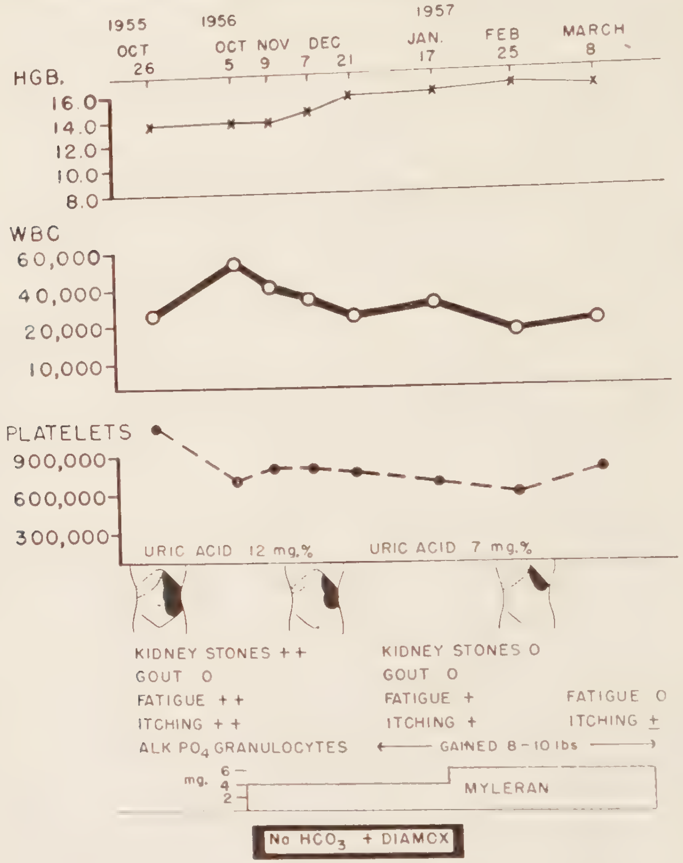

FIGURE 3. *Case J. Z.* Myelosclerosis-myeloid metaplasia. High blood uric acid wit renal calculi. Therapy with Myleran induced reduction in spleen size, reduction leukocyte and platelet counts, and a rise in red cell count. The blood uric acid w reduced. Alkalinization of the urine with the use of sodium bicarbonate and of acetazol mide (Diamox) at night resulted in cessation of uric acid stone formation.

of chronic granulocytic leukemia (FIGURE 1). This might indicate repop lation of the marrow with normal granulocytes and perhaps "cure" of th leukemic process.

Myelofibrosis with myeloid metaplasia of the spleen presents a difficu therapeutic problem. The large bulky spleen is often very distressing the patient and is furthermore productive, in some cases, of an excessi degree of hemolysis. Anemia may become outstanding, not alone because of the sclerotic bone marrow, but through increased hemolysis. We have tried various measures to cope with this problem, including X-ray therapy over the spleen, splenectomy, corticosteroids and, more recently, Myleran. In our experience X-ray therapy has usually done more harm than good; the spleen usually shrinks to some extent, but anemia becomes quite marked. Within a month or two, the spleen has returned to its original enlarged size, so that little if anything has been gained. In occasional instances, when one of the corticosteroids was given prior to projected splenectomy, a striking and at times a lengthy remission took place, indicating that a trial with this material may be indicated. Splenectomy has been performed in 8 cases;

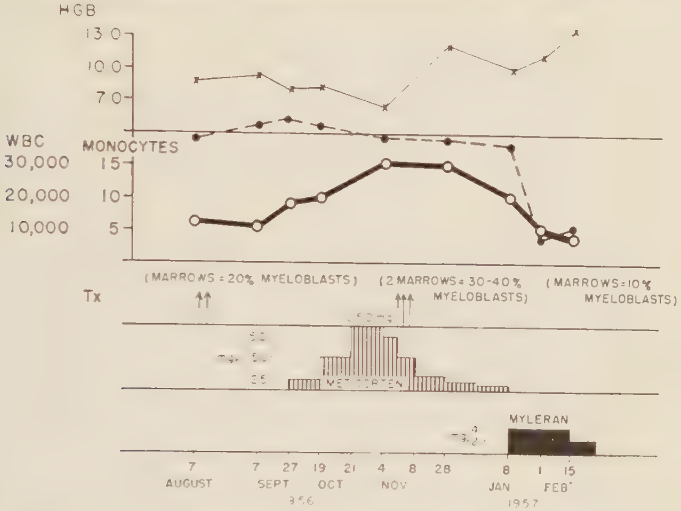

FIGURE 4. *Case P. C.* Subacute granulocytic leukemia. Therapy with Myleran in small doses resulted in striking clinical improvement with improvement in the blood picture. A rise in hemoglobin, a fall in leukocyte count, and a marked reduction in the concentration of primitive white cells of the marrow took place.

n 2 cases there was a rather quick postoperative mortality associated with an extreme thrombocytosis and the development of numerous thromboses. In 2 cases no definite results occurred, but in 4 instances remissions of 6 to 24 months took place, with improvement of hemoglobin and red cell values and a marked diminution in transfusion requirements. These were cases n which, in addition to a hemolytic component, thrombocytopenia was resent; following splenectomy, the platelets returned to somewhat elevated ut not excessively high levels. More recently, we have been using Myleran n the attempt to reduce the "marrow" tissue of the spleen. In 4 of 8 cases, triking effects took place, with marked reduction of spleen size, marked owering of the leukocyte count, and either no modification or else improvenent in the erythrocyte count (FIGURES 2 and 3).

Due to the rather striking effects of Myleran in myeloid metaplasia, and ecause of its value as a bone marrow "depressant," we have recently been using it in cases of polycythemia vera in place of P^{32}. It is too early as yet to assess the results of treatment accurately, but beneficial results are already being obtained. Substitution of the alkylating agent Myleran for the ionizing radiations of P^{32} may be advisable, even though, like X ray, Myleran is a radiomimetic and probably mutagenic agent. Thus, the possibility of inducing acute leukemia is present, although this seems less likely than with the ionizing radiations, which are known leukemogenic agents.

In the acute and subacute granulocytic leukemias, Myleran has not ordinarily been helpful, but in one of our adult cases previously treated with corticosteroids with little effect, a rather striking remission took place when Myleran was given (FIGURE 4).

Summary

The cells of the bone marrow are subject to proliferation, either in a total manner or selectively, and either in a relatively benign chronic form or in a more malignant acute form. The myeloproliferative disorders include such conditions as polycythemia vera, chronic granulocytic leukemia, myelofibrosis with myeloid metaplasia, thrombocythemia, and various forms of granulocytic leukemia.

Myleran, particularly when given in relatively small dosage, appears to have a more or less selective action on bone marrow cells. Thus, this drug is particularly effective in chronic granulocytic leukemia, in which it may induce very prolonged remissions. It may be a useful drug in some cases of myelofibrosis with myeloid metaplasia. In polycythemia vera, its action as a bone marrow depressant may be useful, thus avoiding the possible leukemogenic effects of radioactive phosphorus.

CHLORAMBUCIL AND TRIETHYLENE THIOPHOSPHORAMIDE IN THE TREATMENT OF NEOPLASTIC DISEASE*

By John E. Ultmann, George A. Hyman, and Alfred Gellhorn
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The usefulness of nitrogen mustard in the therapy of lymphomas prompted intensive investigation of related compounds. Studies by Haddow and his associates[1] [2] led to the development of aliphatic and aromatic nitrogen mustards such as Myleran and chlorambucil. Investigation in another direction, prompted by the discovery of the properties of triethylene melamine, led to the synthesis of other ethylenimine compounds, including triethylene thiophosphoramide.[3]

This communication summarizes our experience in the therapy of various neoplastic diseases with chorambucil and with triethylene thiophosphoramide at Francis Delafield Hospital and at Presbyterian Hospital. In the evaluation of these agents, their effect on the particular diseases studied will refer to objective changes only.[4] [5] Objective criteria will necessarily vary in detail depending upon the disease. However, in all instances a favorable objective response to therapy consisted of regression or significant improvement of *all* evident disease without progression in any single area. Application of therapy to local areas and their consequent improvement has not been studied in this series of patients, and our evaluation rested on the total objective response of disseminated disease.

Chlorambucil

Since August 1954, 60 patients have been treated with 67 courses of chlorambucil (*p*-(di-2-chloroethyl)aminophenylbutyric acid, CB 1348). Thirty-two patients had chronic lymphocytic leukemia, 14 lymphosarcoma, 8 Hodgkin's disease, and 6 multiple myeloma. Diagnosis in each patient was established by history, physical examination, and examination of the appropriate hematological and biopsy material.

Chlorambucil was administered by mouth in all cases. For the larger part of the study, enteric-coated tablets of 2- and 5-mg. size were used. Single daily doses averaged about 10 mg., but individual doses ranged between 2 and

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The chlorambucil used in this study was supplied in part by A. Haddow and D. A. G. Galton of the Chester Beatty Research Institute, London, England, and in part by Donald Searle of Burroughs Wellcome & Co., Tuckahoe, N. Y. Triethylene thiophosphoramide was supplied by James Ruegsegger of Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

Jane E. Harvey and Ana Tiburcio gave technical assistance in this study.

30 mg. (TABLE 1), depending on body weight, diagnosis, hematological picture, and previous therapy. The average total dose was 430 mg., with effective dosage between 50 and 1200 mg. The average total dosage on a body-weight basis was 7 mg. per kg., administered over an average period of 58 days. Eight patients have been given maintenance therapy averaging 3 mg. daily over a period of 11 months.

TABLE 1

DOSAGE AND DURATION OF THERAPY WITH CHLORAMBUCIL

	Average	Range
Daily dose	10 mg.	2–30 mg.
Total dose/course	435 mg.	36–1200 mg.
Total dose/kilogram/course	7.1 mg./kg.	0.7–18.0 mg./kg.
Duration of course	58 days	6–200 days
Maintenance (8 patients)	3.0 mg./day for an average of 11 months	

The results of therapy are summarized in TABLE 2. The criteria for objective improvement of these patients have been reported previously.[4] Among the 32 patients with chronic lymphocytic leukemia given 36 courses of treatment, significant objective improvement occurred in 21 instances, lasting an average of 8 months. In these patients, in all instances, the white blood cell count fell to less than 25 per cent of initial values, 10 patients reachin 10,000 white blood cells/cu. mm. or less. However, the percentage of lymphocytes was decreased by 25 per cent or more in only 8 cases, and to 40 per cen lymphocytes on differential in only 3. A significant decrease of hepatosplenomegaly and lymphnode enlargement followed 15 courses of therapy In the 14 patients with lymphosarcoma (including 12 with lymphocyt lymphosarcoma and 1 each with reticulum cell sarcoma and giant follicul

TABLE 2

RESULTS OF THERAPY WITH CHLORAMBUCIL IN 61 PATIENTS (2/28/57)

Diagnosis	Patients (number)	Courses (number)	Objective response and duration (months)				
			Exc.—Rem.	(mo.)	Ptl.—Rem.	(mo.)	None
Chronic lymphocytic leukemia	32	36	4	11	17	7	15
Lymphocytic lymphosarcoma	14	15	1	4	6	12	8
Hodgkin's disease	8	10	0	—	2	4	8
Multiple myeloma	6	6	0	—	0	—	6
Total	60	67	5	—	25	—	37

lymphosarcoma) given 15 courses of treatment, 7 showed significant improvement, lasting approximately 10 months. It is of interest that these patients with predominantly tissue involvement by lymphosarcoma required on the average 150 mg. more chlorambucil over an additional 17 days to achieve maximal benefit comparable to that of the patients with chronic lymphocytic leukemia. In 8 cases of Hodgkin's disease, only 2 showed partial temporary, objective improvement in spite of doses of chlorambucil adequate to produce moderate leukopenia. In 6 patients with multiple myeloma, although cautious trial with chlorambucil resulted in no beneficial effects, the small doses were sufficient to cause additional transient hematopoietic depression in 4 patients.

The drug was administered orally without producing gastrointestinal complaints. There were no instances of hepatic or renal complications except for one patient with chronic lymphocytic leukemia who developed hyperuricemia and transitory oliguria. In the patient with giant follicular lymphosarcoma, who had been treated previously with radiotherapy, a course of 140 mg. (2.3 mg./kg.) administered over 2 weeks resulted in severe transient leukopenia and thrombopenia complicated by pneumonia and purpura. In the patients with multiple myeloma, all of whom had previous radiotherapy and or urethan, moderate doses of chlorambucil (averaging 4 mg./kg./course) resulted in further depression of hematopoiesis.

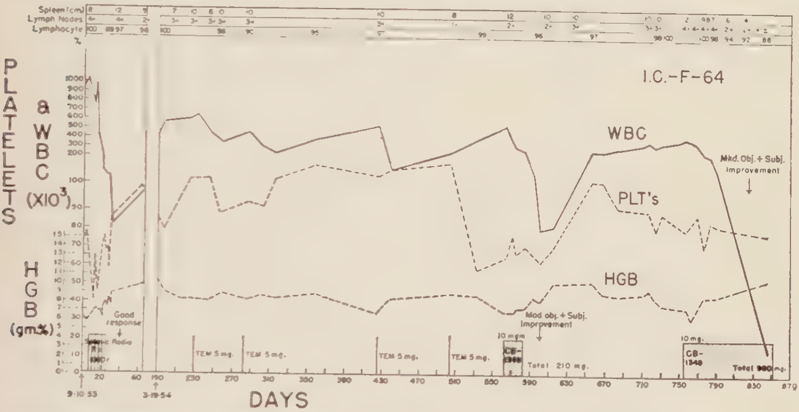

FIGURE 1. Course of a 64 year old woman with chronic lymphatic leukemia. She had an excellent response to splenic radiation. She failed to respond to triethylene melamine (TEM), 5 mg. on 4 occasions, but eventually developed marked thrombopenia. After a significant improvement on two courses of chlorambucil, she has been in remission on 3 mg. daily maintenance of chlorambucil for 14 months.

Fourteen of the 32 patients with chronic lymphocytic leukemia had initial direct platelet counts below 100,000 per cu. mm. of blood at the start of therapy. With effective courses of chlorambucil, carefully administered, the platelet levels did not fall significantly in any of these patients, and actually rose in some, coincident with a fall in the white blood cell count. One patient (I. C., FIGURE 1) who had little response to triethylene melamine (TEM) on

4 occasions, but had developed thrombocytopenia (58,000 per cu. mm.), showed significant response to 2 courses of chlorambucil with some rise in platelet count. This patient remains in remission on maintenance chlorambucil 14 months after completion of the second course.

The wide range of effective dosages required by different patients (rarely as low as 1 mg. kg. course) required cautious administration initially. Because of this the period of therapy with chlorambucil was considerably longer than with triethylene melamine. In one patient (M. L., FIGURE 2), for instance, the administration of single doses of TEM resulted in remissions of 1 year on 2 occasions. In contrast, chlorambucil therapy required nearly 2 months to achieve a similar response. This patient now has been in remission without maintenance therapy for 14 months.

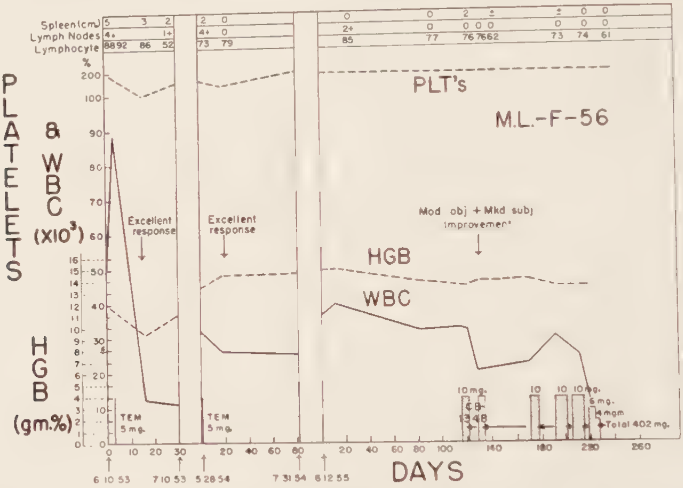

FIGURE 2. In this 56-year-old woman with chronic lymphatic leukemia, 2 single doses of triethylene melamine (TEM) resulted in remissions of 1 year each. In contrast, chlorambucil therapy required nearly 2 months to achieve a similar response. The chlorambucil-induced remission has lasted 14 months without maintenance therapy.

The current therapeutic program, which has proved safe in symptomati patients with chronic lymphocytic leukemia, consists of a trial of 70 to 10 mg. of chlorambucil given over a week, the amount depending on hematological status and previous therapy. As the second week of therapy frequently reveals the unusually sensitive patient, the dose of chlorambucil i either reduced or the patient is seen more frequently. After this period, i the white count remains elevated, the patient is given 6 to 10 mg. of chlorambucil daily and is observed at least weekly with appropriate blood studies including a platelet count. Mild hematopoietic depression is rarely seriou and reverses promptly upon the withdrawal of chlorambucil. When a optimal blood count (5000 to 10,000 white blood cells cu. mm.) has bee reached, the drug is continued in smaller daily doses until maximal regressio of lymph nodes and hepatosplenomegaly are achieved, at which time the dru is stopped. Currently a number of patients who suffered a relapse have been retreated and then placed on maintenance therapy consisting of from 2 mg. every other day to 5 mg. daily. With lymphocytic lymphosarcoma, when generalized and previously untreated, our approach is more aggressive after the usual therapeutic trial of about 100 mg. in the first week. To avoid renal shutdown following hyperuricemia, all patients are encouraged to drink from 3000 to 4000 ml. of fluids daily during the course of treatment.

Triethylene Thiophosphoramide

Our experience with triethylene thiophosphoramide (N,N′,N″-triethylene thiophosphoramide, Thio-TEPA, S-TEPA) has been reviewed recently.[5] Since 1953, 100 patients with biopsy-proved malignant neoplasms have received 112 courses of Thio-TEPA. The diagnoses, number of patients, and the number of courses they received are shown in TABLE 3.

TABLE 3
RESULTS OF THERAPY WITH THIO-TEPA IN 100 PATIENTS (2/28/57)

Diagnosis	Patients (number)	Courses (number)	Objective response		
			Exc.	Ptl.	None
Hodgkin's disease	13	16	3	6	7
Lymphosarcoma	30	33	1	9	23
Carc. ovary	27	33	4	12	17
Misc. carcinomas	25	25	0	0	25
Malignant melanoma	5	5	0	0	5
Total	100	112	8	27	77

Triethylene thiophosphoramide was prepared from the crystalline powder in sterile isotonic saline solutions to make a final concentration of 10 mg./ml. Daily doses, averaging 10 mg., were administered over a few days. Thio-TEPA was given intravenously to 91 patients. Two patients received intramuscular and 2 intra-arterial injections. The remaining 5 patients received single intracavitary injections of 20 to 50 mg. each. The average course was 50 mg., usually given over 7 days, the total dose averaging 1.2 mg. per kg. TABLE 4 shows the average and range of the dosage of Thio-TEPA employed. Not included in the range are a patient who received 215 mg. over a 5-month period and another patient who received 360 mg. in a year.

The criteria employed in our evaluation of objective changes in this group of patients have been described in detail elsewhere[1, 5] and will necessarily vary with each disease. In the lymphoma group, including Hodgkin's disease and lymphosarcoma (TABLE 3), significant objective improvement was obtained in 19 of 49 courses given to 43 patients. These changes generally were of short duration; only 3 patients had remissions of 6 months or longer. FIGURE 3 (patient C. H.) compares the responses of a patient with

Hodgkin's disease to treatment with radiotherapy, with nitrogen mustard, and with Thio-TEPA on 2 occasions. Each treatment resulted in a decrease of temperature to normal, weight gain, and diminution in the size of the spleen and lymph nodes; each time remissions lasted about 200 days. FIGURE 4 (patient A. R.) illustrates the effect of Thio-TEPA in a patient with reticulum-cell sarcoma. After an initial severe leukopenia and thrombopenia, the tumor responded almost completely, except for a residual spleen tip. The patient remained at first in complete, later in partial, remission for 8 months and died following failure to respond to Meticorten (prednisone) and a second course of Thio-TEPA.

TABLE 4
DOSAGE AND DURATION OF THERAPY WITH THIO-TEPA

	Average	Range
Daily dose	10 mg.	3.5–20 mg.
Total dose/course	50 mg.	30–115 mg.
Total dose/kilogram/course	1.2 mg./kg.	0.4–3.5 mg./kg.
Duration of course	7 days	

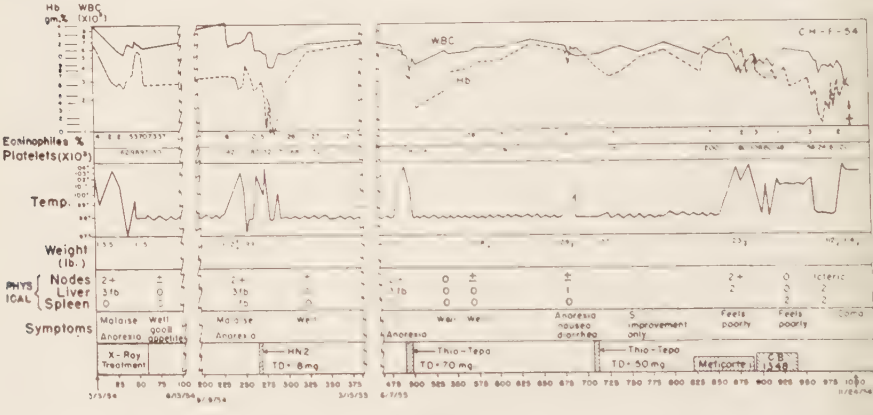

FIGURE 3. Course of a 54 year-old woman with Hodgkin's disease, comparing th responses to therapy with X ray, nitrogen mustard, Thio-TEPA, and chlorambucil. O 3 occasions the patient showed excellent objective improvement. Toward the end of he course she failed to respond completely to a second course of Thio TEPA and showed n response to Meticorten or to chlorambucil.

In 25 patients with various carcinomas, including 9 with carcinoma of th breast and 5 with malignant melanoma, no significant objective improvement was observed (TABLE 3). However, there was significant improvemen in 14 of 27 patients with carcinoma of the ovary given 33 courses of Thio TEPA. TABLE 5 summarizes the initial therapeutic response of patient

TABLE 5
EFFECT OF THIO-TEPA ON OVARIAN CANCER (2/28/57)

	No. of pts.	Objective response and survival (months)								
		Exc.	Died (mo.)	Alive (mo.)	Ptl.	Died (mo.)	Alive (mo.)	None	Died (mo.)	Alive (mo.)
Papillary.....	11	1	20		5	7.9	16*	5	2.5	
Pseudomuc...	2							2	4.5	
Adenoca......	5				2	10	14*	3	1.8	
Undiff........	6	2	19	15*	3	4.7		1	0.3	
Unclass.......	3	1	12					2	1.1	
Total......	27	4	17	15	10	7.5	15	13	2.2	0

* One patient.

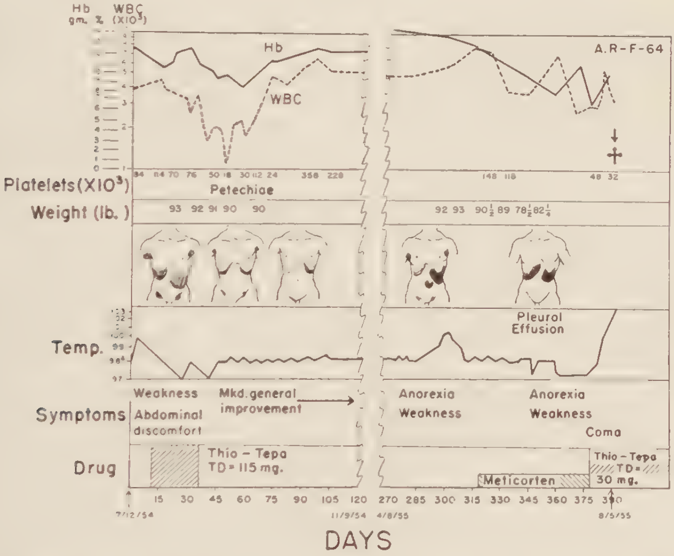

FIGURE 4. Sixty-four-year-old woman with disseminated reticulum-cell sarcoma who showed marked objective improvement after a course of Thio-TEPA. During therapy there was severe leukopenia and thrombopenia, associated with transient purpura. After a remission of eight months, the patient relapsed and failed to respond to Meticorten and a second course of Thio-TEPA.

according to the histological classification. Six of 11 patients with papillary adenocarcinoma and 5 of 6 patients with undifferentiated carcinoma showed significant objective improvement with decrease or disappearance of pleural effusions, ascites, and/or abdominal masses. FIGURE 5 (patient S. S.) shows a patient with biopsy-proved carcinoma of the ovary who showed complete disappearance of pleural and abdominal fluid after 60 mg. of Thio-TEPA given intravenously. On small doses of maintenance therapy this patient remained essentially asymptomatic for 8 months. At that time, masses were felt in 1 breast and, on biopsy, a separate, histologically distinct carcinoma of the breast was discovered. This tumor did not respond to systemic Thio-TEPA therapy, and the patient died. At autopsy, there were found only a few residual foci of carcinoma cells from the ovarian tumor with mainly fibrous replacement; widespread carcinoma of the breast was demonstrated with no cytological changes suggesting drug effect.

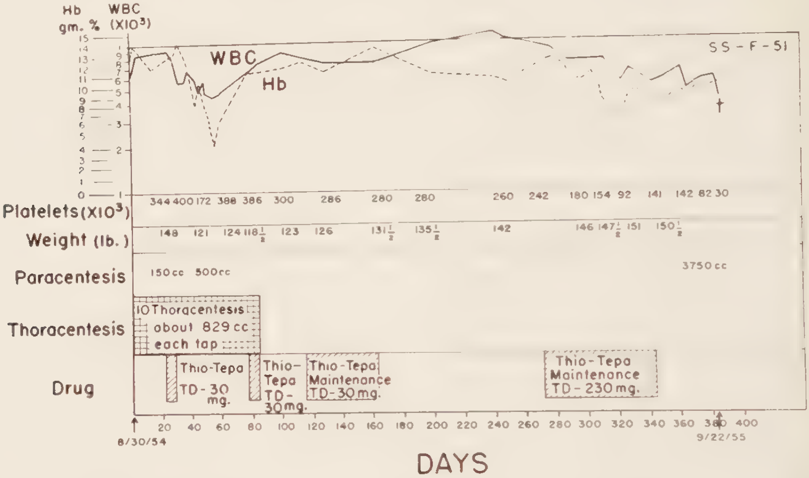

FIGURE 5. Fifty-one-year-old woman with carcinoma of the ovary who was completely relieved of recurrent pleural and abdominal effusions after a course of intravenous Thio-TEPA. On maintenance with Thio-TEPA the patient remained asymptomatic for eight months. When she relapsed, breast and axillary masses appeared that proved, on biopsy, to be a carcinoma of the breast and that did not respond to further chemotherapy with Thio-TEPA.

The duration of remission in the group of treated patients with carcinoma of the ovary was from 2 weeks to 20 months. The mean survival time (TABLE 5) from onset of therapy for those patients who failed to show improvement was 2.2 months, with no patient surviving. Among the 14 patients who showed initial improvement, 3 are still living 14, 15, and 16 months after initiation of therapy, while the 11 who have died survived an average of 13.0 months. It would appear that effective treatment significantly modifies the natural history of carcinoma of the ovary.

Administration of Thio-TEPA by various routes caused no local or immediate systemic reaction. In the 100 patients to whom 112 courses of therapy were administered, 47 showed hematological depression. Platelets alone were depressed below 75,000 cu. mm. in 14 instances, white blood cells alone were below 2000 cu. mm. in 5 instances, and both were decreased in 28 cases. Platelet depression occurred not only more frequently, but generally appeared later and persisted longer than leukopenia. In 17 patients, hematopoietic toxicity was accompanied by clinical manifestations with complications of thrombopenia 14 times and with evidence of infection 11 times. The onset of toxicity usually did not appear until 19 days after initiation of therapy. In patients with poor hematological status at the start of therapy, or with a history of previous chemotherapy, or with disseminated lymphoma, the onset of toxic manifestations appeared earlier and generally lasted longer (TABLE 6). Two patients with disseminated reticulum cell sarcoma developed further hyperuricemia during therapy and died with irreversible renal shutdown.

Currently, we recommend Thio-TEPA only for patients with disseminated ovarian carcinoma. The initial course usually consists of 40 mg. of Thio-TEPA given intravenously over the period of a week. An additional 10 or 20 mg. may be given after a period of 3 weeks has elapsed if the blood count has not changed remarkably. Maintenance therapy is not given to patients with a falling white blood cell count or falling platelet count, but may be given cautiously to patients when the blood count has risen to stable low normal values.

TABLE 6

ONSET AND DURATION OF TOXICITY AFTER THIO-TEPA THERAPY IN 47 PATIENTS

	Hematological status			Previous therapy			Diagnosis		
	Exc.	Good	Poor	None	X Ray or Au 198	Chemo. c̄/o X ray	Ca. Ov.	Retic. Sa.	HD
Onset of toxic manifestation from start of daily therapy (average days)	22	18	12	20	20	17	25	22	12
Return of abnormal blood counts to normal from start of toxicity (average days)	18	31	35	33	18	23	15	34	31

Summary

Chlorambucil was administered by mouth in doses ranging from 2 to 30 mg. daily for an average of 8 weeks. In the largest group, 32 patients with chronic lymphocytic leukemia given 36 courses of treatment, significant objective improvement occurred in 21 instances, lasting an average of 9 months. In 14 patients with lymphocytic lymphosarcoma, significant improvement was noted in 7. In Hodgkin's disease, the results were less encouraging while, in multiple myeloma, no improvement was obtained. In the average dosage recommended (7 mg./kg. or about 430 mg./course),

chlorambucil was practically without side effects. It was particularly useful in patients with modest thrombopenia, where other agents had proved hazardous.

The effects of 112 therapeutic courses of triethylene thiophosphoramide in 100 patients with various disseminated malignant neoplasms have been reported. The average course was 50 mg. given intravenously over 5 to 7 days. Significant objective changes were obtained in 19 of 49 courses given to 43 patients with malignant lymphomas, but these were generally of short duration. In 57 patients with various carcinomas and with malignant melanoma, significant objective improvement was observed only in those with ovarian carcinoma. Fourteen of 27 patients with the latter tumor were objectively benefited, and the survival time of these patients was prolonged significantly when compared with that of the patients in whom the therapy failed. Delayed and sometimes severe hematological depression occurred in 43 instances and, accompanied by clinical manifestations in 17 patients, coupled with the unpredictability of the dosage-toxicity relationship and the transitory effect, outweighed the therapeutic value of Thio-TEPA except in ovarian carcinoma.

References

1. Haddow, A. 1953. Mode of action of carcinogenic agents on cells. Acta Unio Intern. contra Cancrum. **9**: 675–680.
2. Galton, D. A. G., L. G. Israels, J. D. N. Nabarro & M. Till. 1955. Clinical trials of *p*-(di-2-chloroethylamino)-phenylbutyric acid (CB 1348) in malignant lymphoma. Brit. Med. J. **2**: 1172–1176.
3. Sugiura, K. & C. C. Stock. 1952. The effect of methylbis(βchloroethyl)amine oxide, N-N′-N″-triethylene phosphoramide and N-N-diethyl-N′-N″-diethylene phosphoramide on the growth of a variety of mouse and rat tumors. (Abstr.) Cancer Research. **12**: 300.
4. Ultmann, J. E., G. A. Hyman & A. Gellhorn. 1956. Chlorambucil in treatment of chronic lymphocytic leukemia and certain lymphomas. J. Am. Med. Assoc. **162**: 178–183.
5. Ultmann, J. E., G. A. Hyman, C. Crandall, H. Naujoks & A. Gellhorn. 1957. Triethylenethiophosphoramide (Thio-TEPA) in the treatment of neoplastic disease. Cancer. **10**: 902–911.

p-(DI-2-CHLOROETHYL)AMINOPHENYLBUTYRIC ACID (CB 1348) IN THE TREATMENT OF CANCER

By Kenneth B. Olson

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A total of 11 cases of malignant lymphoma, chronic lymphatic leukemia, and myeloma have been treated from 6 to 60 days with *p*-(di-2-chloroethyl)-aminophenylbutyric acid (CB 1348).* In most cases the treatment period was 21 days, unless leukopenia occurred. The usual dose was 0.2 mg. per kg., or from 10 to 14 mg. per patient per day, orally.

Five of 7 patients with Hodgkin's disease experienced short regressions for a duration of 1 to 2 months, with subjective improvement as well as diminution in size of the lymph nodes and spleen. These 5 cases all developed marked leukopenia, anemia, and thrombocytopenia, and most required blood transfusions and were given cortisone or prednisone. Remissions lasted from 4 to 6 weeks and, in 4 cases, retreatment was without effect. All cases had been treated previously with roentgenotherapy and nitrogen mustard. One patient developed fever and a diffuse erythematous rash after 6 days of treatment. The symptoms and rash subsided rapidly on withdrawal of the drug, and sensitivity was suspected. A skin biopsy indicated a nonspecific dermatitis.

One patient with a diagnosis of multiple myeloma experienced marked relief of pain and some improvement of bone marrow findings. This remission has persisted for 8 months, but prednisone therapy may well have contributed to the good result. The patient was unable to tolerate urethan.

Two cases of chronic lymphatic leukemia were treated. One case that had failed to respond to total-body radiation responded well to CB 1348, but developed severe anemia. Retreatment has had little effect. Another case did not respond to a 20-day course of CB 1348, but has done well with cortisone. This last case developed severe aplasia of the bone marrow.

CB 1348 appears to be of value in the treatment of lymphatic leukemia and myeloma. Remissions in Hodgkin's disease were transient, and complications of treatment seemed so severe that the over-all benefit to patients was not evident.

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* The drug used in these studies was kindly supplied by A. Haddow of the Chester Beatty Institute, Royal Marsden Hospital, London, England.

THE TREATMENT OF ADVANCED CANCER WITH TRIETHYLENE THIOPHOSPHORAMIDE

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Triethylene thiophosphoramide (Thio-TEPA)* was first used clinically in the treatment of leukemia by Shay *et al.*[1] and subsequently has been used by a number of investigators in the treatment of a variety of types of cancer. This alkylating agent does not produce an immediate reaction, but may cause an early or a delayed depression of the hematopoietic system. This study was undertaken in an attempt to evaluate this material and to determine whether it is superior to other similar agents now in use, especially nitrogen mustard (HN2).

Methods and Materials

Thio-TEPA was received in powder form and was dissolved to give a solution containing 10 mg. per ml. Originally this solution was filtered through a sintered glass filter and placed in sterile ampoules. Sterility was determined after 4 days by means of culture. Subsequently, it was learned that this material was self-sterilizing and, in the latter part of the study, filtration was omitted. In most instances, Thio-TEPA was given by an intravenous route, but it was also administered orally, subcutaneously, intramuscularly, intraperitoneally, and intrapleurally, and was injected directly into tumor masses. By injection, the dose was 10 mg. every other day for a usual treatment course of 4 doses. When the material was given by mouth, the dose was still 10 mg. per day, but it was continued for longer periods and was given daily. Hemograms were obtained before each parenteral dose and at least twice per week when administered orally. Occasionally, administration was continued until a tumor response was obtained or until leukopenia developed.

Forty-five patients with advanced cancer were treated. Histologica evidence of tumor had been established in all cases. All cases had receive prior treatment with roentgenotherapy, nitrogen mustard, hormones, or a combination of these.

Results

Table 1 summarizes the results of treatment and response in 45 cases with 15 different histological types of tumors.

Malignant lymphoma. This group consisted of 9 patients with Hodgkin' disease and 2 with lymphosarcoma. Seven patients had subjective relie that consisted of relief of pain and improvement of appetite; 5 noted a decrease in the size of the tumor masses, a return of body temperature t normal levels, and an increase of body weight. The duration of remission

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* We are indebted to James Ruegsegger, Lederle Laboratories Division, America Cyanamid Company, Pearl River, N. Y., for the supplies of triethylene thiophosphoramid

Table 1

Triethylene Thiophosphoramide in the Treatment of Advanced Cancer

Primary site	Cases	Avg. dose (mg.)	Dose range (mg.)	Subj. imp.	Obj. imp.	Leukopenia
Lymphoma	11	91	25–300	7	5	10
Breast	10	41	40–80	3	2	7
Lung	6	71	40–180	0	0	3
Colon and rectum	4	52	30–80	2	0	1
Ovary	2	45	40–50	0	0	0
Larynx	2	65	40–90	0	0	0
Wilms' tumor	2	40	40	1	1	1
Bladder	1	55	40–80	1	0	1
Thyroid	1			0	0	0
Lymph. leukemia	1			1	1	1 (died)
Kidney	1			0	0	0
Tongue	1			0	0	0
Melanoma	1			0	0	1
Ewing's tumor	1			1	0	1
Squamous cell of the buttock	1			1	1?	1
Total				17 (37%)	10 (22%)	27 (60%)

was 6 months in 1 patient, but was less than 2 months in the remainder, and averaged 7.1 weeks. In general, the remissions were partial and brief. In a previous review[2] it has been noted that when Hodgkin's disease was treated initially with roentgenotherapy, 71 per cent of the patients obtained objective remissions; when the initial treatment was with HN2, the remission rate was 85 per cent. With subsequent treatments with HN2 or roentgenotherapy, approximately 65 per cent of courses of treatment produced objective remissions. By these standards, the remission rate of 45 per cent did not appear to be as good as that obtained by either roentgenotherapy or HN2. The small number of cases does not permit statistical conclusions, but certainly there is no evidence that Thio-TEPA is superior to either roentgenotherapy or HN2.

Leukopenia of less than 4000 leukocytes per cu. mm. developed in 10 of the 11 patients and varied markedly in the time of onset. In 4 patients treatment was stopped because of leukopenia when less than a total dose of 40 mg. had been given. The smallest dose that produced severe leukopenia was 25 mg. intravenously. Seven patients developed delayed leukopenia from 7 to 30 days after the cessation of treatment; the average duration from cessation of treatment to the development of a low leukocyte count was 16 days. In 5 patients a severe aplasia of the bone marrow occurred with thrombocytopenia, anemia, and purpura.

Total dosage ranged from 25 to 300 mg. intravenously, but was less than 40 mg. in 7 patients. The average dose was 91 mg., but this was elevated because in 4 patients it was noted that leukopenia did not develop after 100 mg., and these individuals were given additional treatment. One patient received 300 mg. before the leukocyte count was lowered. In addition to intravenous treatment, 1 patient received 660 mg. by the oral route with little or no effect on the tumor or leukocyte counts.

Breast cancer. Ten cases of breast cancer received a total of 12 courses of treatment; the average dose per patient was 41 mg. in a period of 8 days. In 4 cases, Thio-TEPA was given intravenously and, in 3 cases, it was injected directly into the tumor mass or was given intramuscularly.

Three patients showed subjective improvement, and 2 of these showed objective evidence of improvement. All of the patients demonstrating improvement received the medication intravenously. One 42-year-old female who was experiencing progression of bone lesions and increased pain 6 weeks after roentgenotherapy had relief of pain and partial healing of bone lesions after 2 courses of Thio-TEPA. This remission lasted about 4 months. Another 61-year-old female showed, by chest roentgenogram, definite clearing of the left upper lung field (FIGURE 1) and marked diminution in size of axillary tumor masses and regression of lymphedema of the arm after 40 mg of Thio-TEPA intravenously. This remission lasted 2 months and was accompanied by anemia, purpura, and thrombocytopenia. Blood transfusions and prednisone were given after the remission developed and may have prolonged the remission, but did not initiate it.

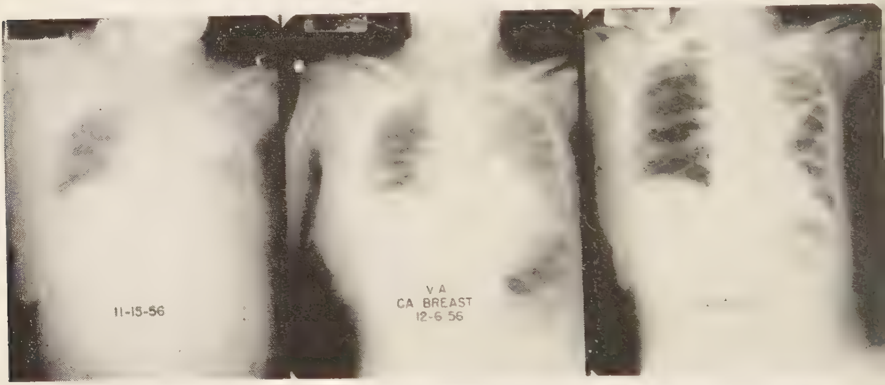

FIGURE 1. Chest roentgenographs on a patient (V. A.) with advanced breast cancer, revealing resolution of infiltration in the left upper lung field 14 days and 39 days after treatment with Thio-TEPA. Concurrently there was diminution in size of tumor masses on the chest wall and in the axilla. Severe aplasia of the bone marrow developed. The dose was 10 mg. intravenously every other day for 8 days.

Seven patients developed leukopenia, and in 2 cases the complications of anemia and thrombocytopenia were severe. In 3 patients leukopenia occurred during treatment, and administration was stopped. In 4 cases leukopenia was delayed and occurred from 14 to 17 days after the last dose. The onset of delayed leukopenia averaged 14.5 days after treatment had ceased. One of 3 patients who received intratumor medication developed a severe leukopenia. None of these 3 demonstrated local or systemic regression of disease.

Bronchogenic carcinoma. Six patients were treated with total doses that ranged from 40 to 180 mg. and averaged 71 mg. Leukopenia occurred in only 1 patient who had been treated intramuscularly with 180 mg., or until leukopenia developed. Four cases were treated by the intravenous route and 2 by intramuscular injection. No subjective or objective improvement was noted.

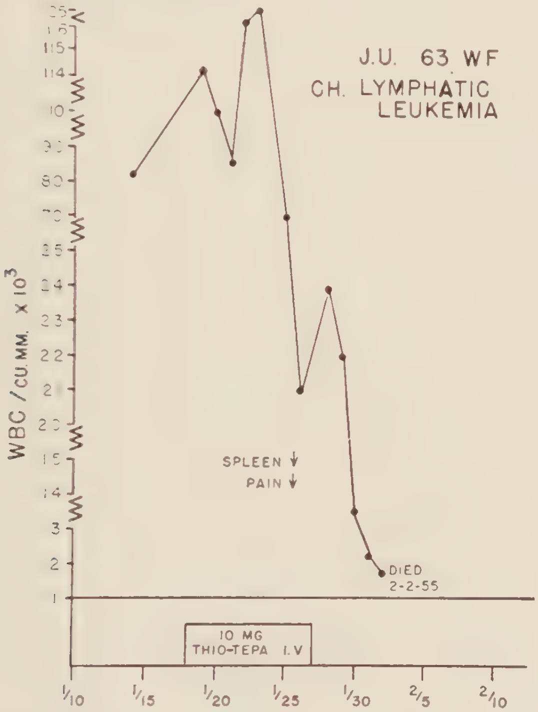

FIGURE 2. Rapid fall of leukocyte count in a case of chronic lymphocytic leukemia treated with 4 daily intravenous injections of 10 mg. of Thio-TEPA. Treatment was terminated when the leukocyte count was 21,000 per cu. mm., but death with severe aplasia of the marrow occurred 7 days later.

Colon and rectum. Four cases were treated with doses of 30 to 80 mg. for an average total dose of 52 mg. One patient developed leukopenia. Two patients each were treated intravenously and intramuscularly. No subjective or objective remissions occurred.

Wilms' tumor. Two patients with embryonal carcinoma of the kidney were treated with a total intramuscular dose of 40 mg. of Thio-TEPA over an 8-day period. One 4-year-old child developed leukopenia 7 days after treatment stopped. This latter child had a remarkable remission that lasted 6 weeks. Metastatic nodules in the lungs cleared almost completely, as demonstrated by roentgenograms, and the child gained weight and experienced a return to normal temperature. Retreatment has been unsuccessful in producing a remission. There was no improvement in the second case.

Cancer of the larynx. Two cases of cancer of the larynx were treated with courses of 40 and 90 mg., respectively. Leukopenia did not develop. There was no improvement noted in either case.

Cancer of the ovary. Two cases were treated because of pleural and peritoneal fluid formation. One case received a single dose of 40 mg. intraperitoneally; in the other case, a dose of 50 mg. in 10-mg. increments was injected into the pleural space over a period of 5 days. There was no decrease in the rate of fluid formation and no evidence of other improvement in either instance.

Lymphatic leukemia. One case of chronic lymphatic leukemia was treated with a dose of 40 mg. in 10 mg. increments injected on 4 successive days; treatment was stopped when the leukocyte count fell from 142,000 to 21,000 per cu. mm. There was marked decrease in the size of the spleen and decrease of fever, but the leukocyte count continued to fall, and the patient died of massive hemorrhages 7 days after the last dose of Thio-TEPA (FIGURE 2).

Miscellaneous. One case each of carcinoma of the bladder, thyroid, kidney, and tongue, and of melanoma, Ewing's tumor, and squamous cell carcinoma of the skin of the buttock were treated. The total dose varied from 40 mg. to 80 mg. and averaged 55 mg. No definite objective improvement was noted in any case, but in 3 there was rather marked decrease of pain. Leukopenia developed in 4 cases, and in 1 of these cases treatment was deliberately continued until leukopenia was noted. In the other 3 cases, leukopenia was delayed; it occurred on an average of 15.5 days after the last dose.

Discussion

Thio-TEPA appears to have a definite antitumor effect that, in this series, was most evident in malignant lymphoma, cancer of the breast, Wilms' tumor, and chronic lymphatic leukemia. For the most part remissions were short, and frequently they were accompanied by complications of severe anemia and thrombocytopenia. There was marked variation in the susceptibility of different individuals in the development of bone marrow depression, and sensitive individuals could not be predicted. Frequent blood studies with termination of drug administration with a falling leukocyte count were ineffectual in preventing serious hematological consequences. In some cases a leukopenia developed during treatment, but in others there was a delayed bone marrow depression that occurred, on the average, about 15 days after cessation of therapy, but varied from 7 to 30 days in individual cases. This emphasizes the necessity for frequent observations for several weeks after treatment has been complete. In no instance was an objective tumor remission noted in the absence of leukopenia.

The results in malignant lymphoma were to be expected and were less frequent than those observed with nitrogen mustard, although the advanced state of the disease in these cases may be an explanation for the failure to observe a higher incidence of response. As it was used, Thio-TEPA seemed to be less rapid in its action than nitrogen mustard and thus has seemed to be a less favorable agent in the severely toxic and febrile patient. There is no evidence presented that it is superior in action to nitrogen mustard, except for the absence of immediate toxicity, and this is counterbalanced by the unpredictability of late bone marrow depression.

The action of Thio-TEPA in breast carcinoma is of interest, but an occasional case showing response has been noted with HN2, and it seems more likely that the results evident here were due to the increased dosage rather than to any specific property of Thio-TEPA. Further study must be conducted in this area. The local action of this material, when injected into superficial breast tumors, metastases, and other lesions, seems rather inconsequential in the whole problem of breast cancer.[3]

Perhaps, as Shay *et al.*[1, 4] originally demonstrated, this agent is of chief value in the treatment of chronic leukemia; with careful titration of the dosage, it would seem that definite results can be produced. The sensitivity of the bone marrow of leukemia patients to alkylating agents makes Thio-TEPA a potentially dangerous as well as a hopeful therapeutic agent.

The dramatic response of one of the cases with a Wilms' tumor is of interest, but similar responses have occurred with the use of nitrogen mustard.

In general, Thio-TEPA has not been shown to be superior to nitrogen mustard, and it requires more frequent observations of the patient and more careful hematological studies if the consequences of severe aplasia of the bone marrow are to be avoided.

Summary

Triethylene thiophosphoramide has been used in the treatment of 45 cases of advanced cancer. Remissions were noted in malignant lymphoma, breast cancer, Wilms' tumor, and chronic lymphatic leukemia. Remissions occurred only with the development of leukopenia, and frequently this was accompanied by thrombocytopenia and anemia that required vigorous treatment. Leukopenia was difficult to predict and occurred sometimes during treatment, but frequently developed several days after treatment. The average onset of delayed leukopenia was 15 days after treatment. Thio-TEPA was not demonstrated to be superior to nitrogen mustard except for its lack of immediate toxic effects.

References

1. Shay, H., C. Zarafonetis, N. Smith, I. Woldow & D. C. H. Sun. 1953. Treatment of leukemia with triethylene thiophosphoramide (thio-TEPA). A.M.A. Arch. Internal Med. **92**: 628–645.
2. Hall, C. A. & K. B. Olson. 1956. Therapy of malignant lymphomas. Am. J. Med. **20**: 392–411.
3. Bateman, J. C. & H. N. Carlton. 1956. Palliation of mammary carcinoma with phosphoramide drugs. J. Am. Med. Assoc. **162**: 701–706.
4. Shay, H. & D. C. H. Sun. 1955. Clinical studies of triethylene thiophosphoramide in the treatment of inoperable cancer. Cancer. **8**: 498–511.

SUMMARY OF RESULTS OBTAINED WITH CB 1348*

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The report that follows represents the results of using *p*-(di-2-chloroethyl)-aminophenylbutyric acid (CB 1348, chlorambucil)† in the treatment of 43 patients with various neoplastic diseases. The background of the development of this chemotherapeutic agent and its effects on Walker carcinoma 256 in rats have been described by Haddow.[1] It is an aromatic alkylating agent with the structural formula illustrated in FIGURE 1.

Galton *et al.*[2] have reported on its efficacy in clinical trial, especially in reference to lymphomas and lymphatic leukemias. The impression gained from this report is that this medication was of value in cases of follicular lymphosarcoma and chronic lymphatic leukemia.

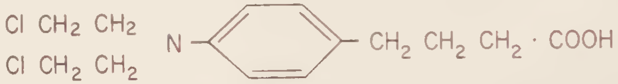

FIGURE 1. *p*-(Di-2-chloroethyl)aminophenylbutyric acid (CB 1348, chlorambucil).

Subsequently, Ultmann *et al.*[3] reported on a series of cases and found that CB 1348 gave the most satisfactory response in cases of chronic lymphocytic leukemia, but was of less value in lymphocytic lymphosarcoma and Hodgkin's disease.

In the cases discussed in this report, CB 1348 was used not only in Hodgkin's disease, leukemia, and lymphomas, but also in a varied assortment of carcinomas.

Since 1955 a total of 43 patients have been treated with CB 1348. A total of 17 of these cases were observed for less than 3 weeks or received less than 150 mg. of medication and thus are not included in this analysis. The remaining 26 cases received what was considered to be an adequate trial of CB 1348 therapy (TABLE 1). The diagnoses and results in these cases are shown on TABLE 2. In these tables, "marked benefit" means objective remission and disappearance of lesions. It is of interest that the best responses were in the cases of lymphosarcoma, Hodgkin's disease, chronic lymphatic leukemia, lymphocytic skin infiltration, and carcinoma of the ovary.

Seven of the 26 cases showed marked improvement (TABLE 3), and an additional 10 showed some benefit. However, the number of individual

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† The CB 1348 employed in this study was furnished through the courtesy of A. Haddow of the Chester Beatty Institute, Royal Marsden Hospital, London, England, and of Burroughs Wellcome & Co., Inc., Tuckahoe, N. Y.

TABLE 1

Case No., name, diagnosis, age, sex	Prior ℞ (X-ray & chemo ℞)	Daily dose, total dose, duration of therapy	Side effects	Present status	Clinical results & comments	Duration improvement*
(1) No. 110 T. C. Leiomyosarcoma (left leg) 43 F	Actinomycin D 1000 γ	4–10 mg. T.D. 462 mg. for 80 days	Slight reduction in WBC to 4200	Living	Leg edema receding. "Recession of edema may be usual postoperative course"	
(2) No. 139 E. G. Carcinoma, breast 66 F		10 mg. T.D. 130 mg. for 13 days	None	Dead	None. Patient died 16 days after CB 1348 started. No toxic effects. Insufficient dose and time to observe	0
(3) No. 159 I. M. Lymphosarcoma 73 F		8 mg. T.D. 64 mg. for 8 days	None	Dead	None. Patient died 8 days after ℞ started. Insufficient dose and time to observe	0
(4) No. 160 M. H. Lymphoma 48 F		10–12 mg. T.D. 110 mg. for 11 days	None	Living	At end of first week "masses seemed smaller." Insufficient time as yet to evaluate. ℞ just started	0
(5) No. 16 J. P. Lymphoma 36 F	Aminopterin, 1949. Bremfoe, 1949. A-Methopterin, 1950. TEM, 1950, 1951, 1952. TEPA, 1953, 1954	2–4 mg. T.D. 117 mg. for 36 days	None	Living	A few small nodes disappeared under ℞ (but preceding biopsy reported only "hyperplasia"). No chemo ℞ in preceding 1 year 3 months. This dose small & duration short. ? if really reappearance	1 year ±
(6) No. 162 S. S. Lymphoma (giant follicular lymphoma) 62 F		10 mg. T.D. 220 mg. for 22 days	None	Living	All cervical, axillary & inguinal nodes have markedly subsided thus far. Partial remission	±
(7) No. 118 M. M. Lymphatic infiltration of skin 36 F	Hydrocortisone, 1955. Meticorten, 1955, 1956. ACTH, 1956. Estrogen (for hot flashes), 1956. X-ray ℞ 19 mo. prior	10 mg. T.D. 490 mg. for 49 days	None	Living	Complete remission. All skin nodules have disappeared & have not returned in 3 months. Meticorten decreased, as CB 1348 given; finally stopped on 11/20/56. CB 1348 stopped on 12/3/56 (WBC, 12/4/56, 4850)	3 months + and still in remission

* Symbols: 0, no effect; ±, slight to moderate beneficial effect; +, marked beneficial effect.

TABLE 1 (*Continued*)

Case No., name, diagnosis, age, sex	Prior ℞ (X-ray & chemo ℞)	Daily dose, total dose, duration of therapy	Side effects	Present status	Clinical results & comments	Duration improvement*	
(8) No. 14 W. M. Chronic lymphatic leukemia 63 M		4–26 mg. T.D. 1242 mg. for 111 days (over period of 122 days)	None	Dead	Nodes, liver & spleen decreased in size. WBC decreased from 276,000 to 81,000. Patient felt much better. Died suddenly 121 days after ℞ started & while improvement appeared to be continuing		+
(9) No. 42 I. B. Malignant melanoma 69 F	TSPA 280 mg. Meticorten, 3955 mg. Actinomycin D 9700 gamma	8 mg. T.D. 24 mg. for 3 days	Marked anemia & death 3 days after CB 1348 started. Probably due to preced. chemo ℞ & tumor	Dead	Left groin mass became smaller. Insufficient time and dose to evaluate		0
(10) No. 72 A. G. Carcinoma, floor of mouth 47 M	Actinomycin D	16 mg. T.D. 112 mg. for 6 days	None	Dead	No improvement. Patient had short, nontoxic dose of CB 1348 between courses of Act. D		0
(11) No. 75 S. A. Carcinoma, ovary (papillary adenocarcinoma) 13 F		8–16 mg. T.D. 934 mg. for 66 days over 91-day period	WBC decreased to 950 after 49 days & 784 mg. CB 1348	Dead	Abdominal mass disappeared after 10 weeks of ℞. Mass did not respond to CB 1348 after it reappeared for second time. TSPA then used	2 weeks after ℞ stopped mass reappeared	+
(12) No. 87 R. T. Lymphosarcoma of skin (mycosis fungoides) 39 F	Nitrogen mustard, 1955. Cortisone, 1956	6–14 mg. T.D. 258 mg. for 18 days	WBC decreased to 4150 after ℞	Living	No improvement. No change after short, nontoxic course of ℞		0
(13) No. 88 J. G. Carcinoma, urinary bladder 68 M		14–18 mg. T.D. 500 mg. for 45 days	None	Dead	No improvement. Nontoxic course of CB 1348 did not appear to affect downhill course of far advanced carcinoma of bladder		0

* Symbols: 0, no effect; ±, slight to moderate beneficial effect; +, marked beneficial effect.

TABLE 1 (*Continued*)

Case No., name, diagnosis, age, sex	Prior ℞ (X-ray & chemo ℞)	Daily dose, total dose, duration of therapy	Side effects	Present status	Clinical results & comments	Duration improvement*
(14) No. 89 V. P. Carcinoma, urinary bladder 72 M	X-ray ℞, 1955. X-ray ℞, 1956	8–16 mg. T.D. 738 mg. for 76 days	WBC decreased to 2550 after 33 days & 640 mg.	Dead	No improvement. No effect from CB 1348. Subsequently cautiously resumed with no effect. Then A-Methopterin given to patient & toxicity & tumor regressed	0
(15) No. 90 F. M. Squamous cell carcinoma, tongue (also pulmonary Tbc) 70 M		8–16 mg. T.D. 416 mg. for 61 days	WBC decreased to 600 after 30 days & 400 mg.	Dead	Submandibular node regressed—tumor in floor of mouth grew	±
(16) No. 43 E. B. Carcinoma, breast 57 F	X-ray ℞, 1955. Actinomycin D, 1956 (5000 gamma)	2–16 mg. T.D. 417 mg. for 35 days	WBC decreased to 900 after 23 days & 352 mg. (Later given with Meticorten, and another drop in WBC occurred)	Dead	No improvement despite marked decreased WBC	0
(17) No. 112 E. C. Carcinoma, ovary (cystadenocarcinoma, also sigmoid adenoca.) 68 F		2 mg. T.D. 2 mg. over 1-day period		Dead	Insufficient dose (only 1 dose of 2 mg. given). Then TSPA 70 mg.	0
(18) No. 119 P. N. Adenocarcinoma, stomach 49 M		10–12 mg. T.D. 644 mg. for 63 days	None	Dead	No improvement. Patient did not become toxic, nor did he improve	0
(19) No. 147 J. DeL. Malignant tumor, type undetermined (plasma cell myeloma?) 57 M		10–14 mg. T.D. 286 mg. for 33 days	WBC decreased to 2350 after 30 days and 230 mg. (patient died 7 days later)	Dead	No improvement. X rays showed progress in bone destruction while on CB 1348	0

* Symbols: 0, no effect; ±, slight to moderate beneficial effect; +, marked beneficial effect.

TABLE 1 (*Continued*)

Case No., name, diagnosis, age, sex	Prior ℞ (X-ray & chemo ℞)	Daily dose, total dose, duration of therapy	Side effects	Present status	Clinical results & comments	Duration improvement*	
(20) No. 127 E. P. Squamous cell carcinoma, hard palate 56 F	Biopsy, July 1954. X-ray, July 1956. Surgical resection, 3/27/56	6–12 mg. T.D. 728 mg. for 70+ days	No toxicity noted	Dead	No improvement. Tumor increased while on CB 1348. CB 1348 not pushed to toxicity		0
(21) No. 105 M. T. Carcinoma, ovary (papillary serous cyst adenoca. right ovary) 63 F		2–14 mg. T.D. 1060 mg. for 180 days	WBC decreased to 3850 after 53 days & 724 mg.	Living	Gained weight. Ascites disappeared. Asymptomatic	180 days	+
(22) No. 133 J. DeR. Adenocarcinoma, 1° site? 50 M	Actinomycin D 13,500 gamma (just prior to CB 1348)	8–14 mg. T.D. 364 mg. for 35 days	No toxicity to CB 1348	Living	No change as yet. Patient also has lupus eryth. CB 1348 still being given		0
(23) No. 99 K. L. Carcinoma, breast 68 M	Actinomycin D 3100 gamma. A-Methopterin, 825 mg. X-ray ℞, 1948–1951. Hormone ℞, 1954–1955	6–10 mg. T.D. 760 mg. for 83 days	No toxicity to CB 1348	Living	No change—lesions not progressing. Appears to be very slow-growing carcinoma of breast. 10 years since mastectomy & 8 years since reappearance		±
(24) No. 13 A. P. Hodgkin's disease 28 F	X-ray ℞, 1952. TEM, 320 mg., 1954. CB 1348, 266 mg., 1954–1955. TSPA, 80 mg., 1955. CB 1348, 1952 mg., 1955–1956. Meticorten, 700 mg., 1956. CB 1348, 1834 mg., 1956–1957	4–20 mg. T.D. 3692 mg. intermittently for 699 days	Mild toxicity. WBC decreased to 4000 once after 2000+ mg. Has since been resumed without toxicity	Living	Masses in groin & axilla regressed under CB 1348. Nodes appear to regress with each course of ℞	Several months with each course of CB 1348	+
(25) No. 40 C. C. Lymphosarcoma 50 M		10–22 mg. T.D. 1208 mg. for 78 days	No toxicity	Lost to study after 78 days of ℞	Small nodule at biopsy site disappeared. No new ones developed	Lost to study	+

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* Symbols: 0, no effect; ±, slight to moderate beneficial effect; +, marked beneficial effect.

TABLE 1 (*Continued*)

Case No., name, diagnosis, age, sex	Prior ℞ (X-ray & chemo ℞)	Daily dose, total dose, duration of therapy	Side effects	Present status	Clinical results & comments	Duration improvement*
(26) No. 68 L. C. Liposarcoma 22 M		8–16 mg. T.D. 216 mg. for 17 days	None	Dead	No improvement. Insufficient time. Only short course of CB 1348—no toxicity. X-ray ℞ then given, with some improvement	0
(27) No. 69 K. H. Carcinoma, prostate 62 M		16 mg. T.D. 208 mg. for 13 days	None	Dead	No change. Insufficient time. Only short nontoxic course of CB 1348 given in 14 days preceding death	0
(28) No. 31 C. A. Chronic myelogenous leukemia 32 M	TSPA, 270 mg., 1955. TEM, 220 mg., 1955 (ended 2 mo. prior to CB 1348)	20–30 mg. T.D. 3054 mg. for 252 days	None	Dead	WBC decreased from 171,000 to eventually 10,000, but then began to rise again. Spleen remained large. Patient never felt very well. Switched to Myleran when WBC rose again. This dropped count, but Hb also, & patient died 10 weeks later	8 months ±
(29) No. 36 E. M. Retroperitoneal fibrosarcoma 50 F	X-ray ℞, 1955	10–26 mg. T.D. 810 mg. for 45 days	WBC decreased to 3800 & platelets to 130,000 after 45 days of ℞. Patient operated upon approximately 2 weeks later & died 2 weeks after this with purpura, leukopenia & few platelets (autopsy—aplastic bone marrow)	Dead	None. Blood dyscrasia after operation possibly attributable to preceding chemo ℞ & X-ray ℞	0
(30) No. 32 S. L. Malignant melanoma 55 M	Chemo ℞ at V.A. Hospital, 1955. X-ray ℞, 1955	8 mg. T.D. 8 mg. for 1 day		Dead	None. Insufficient dose & time	0

* Symbols: 0, no effect; ±, slight to moderate beneficial effect; +, marked beneficial effect.

TABLE 1 (*Continued*)

Case No., name, diagnosis, age, sex	Prior ℞ (X-ray & chemo ℞)	Daily dose, total dose, duration of therapy	Side effects	Present status	Clinical results & comments	Duration improvement*
(31) No. 19 C. B. Malignant melanoma 72 M		8 mg. T.D. 32 mg. for 4 days		Dead	None. Insufficient dose & time. Patient had far advanced disease & died 4 days after ℞ started	0
(32) No. 28 J. L. Neuroblastoma 6 F	HN2 & Meticorten. X-ray ℞ (ended 2 mo. prior to CB 1348)	2 mg. T.D. 28 mg. (with Meticorten) 14 days over 34 days	None	Dead	No improvement. Insufficient time. Small dose—child. No change noted for better	0
(33) No. 15 F. J. Hodgkin's disease 36 M	X-ray ℞, 1944. TEM, 1951–1953. TEPA, 1954. TSPA, 1954–1955	4–16 mg. T.D. 1168 mg. (started 1 mo. after TSPA stopped for 300 days)	WBC decreased to 3250 on one occasion	Living	Large nodes decreased under ℞. Took 10 mo. to produce remission	All nodes gone for 1 mo. now +
(34) No. 91 R. G. Carcinoma, breast 58 F	X-ray ℞, 1955. Actinomycin D, 1956. A-Methopterin, 1956-1957	10–12 mg. T.D. 154 mg. for 14 days		Living	Insufficient time and dose	0
(35) No. 123 M. L. Squamous cell carcinoma, scalp, with cervical node metas. 31 F		10 mg. T.D. 180 mg. over 18 days	None	Dead	None. Patient transferred to X-ray ℞. Insufficient dose and time to evaluate chemo ℞	0
(36) No. 154 M. P. Adenoca., peritoneal cavity, 1° site? 64 F	Actinomycin D, Jan. 1957, 3000 gamma	10 mg. T.D. 100 mg. for 10 days		Living	None. Insufficient time and dose	0
(37) No. 55 A. J. Lymphoma 35 M	TEPA, 1953. Gamma-globulin, 1954-1955	2–10 mg. T.D. 394 mg. for 60 days	Decrease in WBC to 3800 in 42 days & 314 mg. (dropped to 3100 with additional CB 1348)	Living	Temporary disappearance of nodes in neck. Temporary brief remission with CB 1348 sufficient to cause WBC decrease	2–3 weeks ±
(38) No. 17 J. B. Carcinoma, lung 49 M		2–22 mg. T.D. 1432 mg. for 106 days	At end of ℞ WBC decreased to 3800	Lost to study	X-ray reported lung mass slightly smaller. CNS signs & symptoms improved	Lost to study ±

* Symbols: 0, no effect; ±, slight to moderate beneficial effect; +, marked beneficial effect.

TABLE 1 (*Concluded*)

Case No., name, diagnosis, age, sex	Prior ℞ (X-ray & chemo ℞)	Daily dose, total dose, duration of therapy	Side effects	Present status	Clinical results & comments	Duration improvement*
(39) No. 166 K. T. Carcinoma, kidney 75 F		10 mg T.D. 140 mg. for 14 days		Living	Insufficient time and dose	0
(40) No. 161 E. M. Carcinoma, rectum 55 F	X-ray ℞, 1956–1957	6 mg T.D. 24 mg. for 4 days		Living	Insufficient time and dose	0
(41) No. 2 J. B. Lymphosarcoma 60 M	Aminopterin, 1948. Teralpherin, 1949. Aminopterin, 1949. Amino-an-fol, 1949–1950. TEM, 1950–1954	2–8 mg. T.D. 578 mg. for 142 days over 420 days	Decreased WBC to 2450 at end of course of CB 1348	Dead	No improvement. No change with CB 1348. Nodes & abdominal mass persisted	0
(42) No. 6 A. S. Chronic lymphatic leukemia 34 M	TSPA, 1955	2–20 mg. T.D. 1068 mg. for 293 days	WBC decreased to 3500 after 177 days and after 672 mg.	Living	Enlarged liver 3 finger breadths disappeared in 3 mo. In 4 mo. all nodes became small; spleen also regressed. Gained 25 lb. (partial remission). CB 1348 ceased because nodes and liver enlarging. Act. D then started	3 months ±
(43) No. 152 W. S. Hodgkin's disease 28 M		6–12 mg. T.D. 244 mg. for 47 days over 151 days	WBC decreased below 3500 (reported by outside doctor)	Dead	Masses in neck and axilla decreased. X-ray ℞ given 2 mo. after CB 1348 because of decreased WBC and presence of neck masses	±

* Symbols: 0, no effect; ±, slight to moderate beneficial effect; +, marked beneficial effect.

cases of any particular neoplasm in this series was much too small to permit any broad generalizations.

Duration of remission was varied but, in general, it seemed to last several months. Three of our 7 cases are still in remission, 3 are dead, and 1 was lost to study.

In general, the plan was to use the medication until either the neoplasm regressed or toxicity developed. Medication then was stopped.

TABLE 2
RESULTS OF TREATMENT WITH CB 1348

Diagnosis	Total treated	+ Marked benefit	± Some benefit	0 No effect
Lymphosarcoma	5	1	3	1
Hodgkin's disease	3	2	1	0
Chronic lymphatic leukemia	2	1	1	0
Lymphocytic skin infiltration	1	1	0	0
Chronic myelogenous leukemia	1	0	1	0
Leiomyosarcoma	1	0	1	0
Fibrosarcoma	1	0	0	1
Malignant tumor, type ?	1	0	0	1
Carcinoma, ovary	2	2	0	0
Carcinoma, prim. site ?	1	0	0	1
Carcinoma, urinary bladder	2	0	0	2
Carcinoma, breast	2	0	1	1
Carcinoma, tongue	1	0	1	0
Carcinoma, stomach	1	0	0	1
Carcinoma, palate	1	0	0	1
Carcinoma, lung	1	0	1	0
Total	26	7	10	9

Dosage

The dose of CB 1348 was usually started low and increased slowly to between 0.2 and 0.3 mg./kg. by mouth daily. Total dose reached as high as 3054 mg. in 252 days in 1 case, and 3693 mg. in 699 days (intermittently) in another case.

Toxicity developed in 12 of the 26 cases treated (TABLE 4). Manifested by mild to severe leukopenia and/or thrombocytopenia, it developed with a total dose that ranged from 230 mg. in 30 days to 1432 mg. in 106 days. Other patients received larger doses without toxicity. Of the 7 cases showing marked beneficial results, 3 developed no toxicity at all, and 3 only mild toxicity. The case that showed white blood count drop with only 230 mg. had bone involvement with "a malignant tumor, type undetermined" (probably plasma cell myeloma), and no preceding X ray or chemotherapy. Possibly the bone involvement may have accounted for the early leukopenia. Of the 14 cases that showed no toxicity, many had had large amounts of radiation and chemotherapy preceding CB 1348 administration but did not develop leukopenia.

One case (C. R. No. 36) with a fibrosarcoma died with leukopenia, thrombocytopenia, purpura, and anaplastic bone marrow 4 weeks after the last dose of CB 1348 and 2 weeks after palliative removal of the 18-lb. tumor had been accomplished surgically. The patient had received a total dose of 810 mg. in 45 days. This death was considered probably due to CB 1348, although large amounts of X-ray therapy also had been administered.

Table 3

Analysis of 7 Cases Showing Marked Benefit from CB 1348

Name C. R. number Diagnosis	Ind. dose Total dose	Toxicity	Comment
A. P. No. 13 Hodgkin's disease	4–20 mg. intermittently over 699 days. T. D. 3692 mg.	Mild. WBC down 4000 after 2000 mg. Since resumed without toxicity	Masses in groin & axilla regressed after each of several courses of ℞. Return in several months. Living
F. J. No. 15 Hodgkin's disease	4–16 mg. over 300 days. T. D. 1168 mg.	WBC down to 3250 once	Took 10 months to produce remission. All nodes gone now. Living
C. C. No. 40 Lymphosarcoma	10–22 mg. over 78 days. T. D. 1208 mg.	0	Patient lost to follow-up after 78 days of ℞. Asymptomatic then. Nodes gone behind angle of jaw
W. M. No. 14 Chronic lymphatic leukemia	4–26 mg. 111 days over 122 days. T. D. 1242 mg.	0	Nodes, liver, spleen down in size. WBC 276,000 to 81,000. Patient felt better. Died suddenly 121 days after ℞ started
M. M. No. 118 Lymphocytic skin infiltration	10 mg. over 49 days. T. D. 490 mg.	0	Complete remission—all skin nodules gone. Now 3 months and still in remission
S. A. No. 75 Carcinoma, ovary	8–16 mg. 66 days over 91 days. T. D. 934 mg.	WBC down to 950 after 49 days & 784 mg.	Mass in abdomen disappeared on CB 1348. It then reappeared 2 weeks after CB 1348 stopped & did *not* respond to second course of ℞
M. T. No. 105 Carcinoma, ovary	2–14 mg. over 180 days. T. D. 1060	WBC down to 3850 after 53 days & 724 mg.	Living, comfortable & no palpable mass or ascites

It was found that, after toxicity had developed and then subsided, CB 1348 could be resumed safely in most of our patients.

Conclusions

(1) From this small series it would appear that CB 1348 may exert a emporary beneficial effect on patients with certain neoplastic diseases.

(2) The cases materially benefited in this series were those with the diagnosis of Hodgkin's disease, lymphosarcoma, chronic lymphatic leukemia, ymphocytic skin infiltration, and carcinoma of the ovary.

TABLE 4

PATIENTS SHOWING MODERATE OR MARKED LEUKOPENIA

Case number	Dose (mg.)	Days of treatment	White blood count
75	784	49	950
89	640	33	2550
90	400	30	600
43	352	23	900
147	230	30	2350
105	724	53	3850
36	810	45	Severe leukopenia, thrombocytopenia & death after subsequent operation
55	314	42	3800
17	1432	106	3800
2	578	142 days of treatment in 420 days	2450
6	672	177	3500
152	244	47 days of treatment in 151 days	Below 3500 (reported by outside doctor)

(3) Dosage of CB 1348 is variable.

(4) CB 1348 therapy may be resumed safely even after it has produced toxicity.

(5) Production of toxicity is not necessary to obtain a beneficial result.

(6) One death was reported that may possibly have been due to the use of CB 1348.

Acknowledgment

We thank Celia Hindin for her technical assistance.

References

1. HADDOW, A. 1954. Experimental and clinical aspects of the action of various carboxylic acid derivatives in the aromatic nitrogen mustard series. Ciba Foundation Symposium on Leukaemia Research. : 196–204. G. E. W. Wolstenholme & M. P Cameron, Eds. Little, Brown. Boston, Mass.
2. GALTON, D. A. G., L. G. ISRAELS, J. D. N. NABARRO & M. TILL. 1955. Clinical trials of p-(di-2-chloroethylamino)-phenylbutyric acid (CB 1348) in malignant lymphoma Brit. Med. J. **2:** 1172–1176.
3. ULTMANN, J. E., G. A. HYMAN & A. GELLHORN. 1956. Chlorambucil in treatment o chronic lymphocytic leukemia and certain lymphomas. J. Am. Med. Assoc. **162:** 178–183.

MYLERAN IN THE TREATMENT OF LYMPHOMAS*

By Marguerite P. Sykes

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Although the therapeutic effect of 1,4-dimethanesulfonoxybutane (Myleran) on chronic myelogenous leukemia[1] has been well established, my associates and I believed that Myleran might be effective also in the lymphomas and chronic lymphatic leukemia, as are other alkylating agents. The purpose of this study was *not* to work up or evaluate a large number of patients, but only to try to obtain complete remissions in the lymphomas.

The first patients in the series received varying daily doses of Myleran ranging from 2 to 10 mg. In the latter cases it was thought to be of importance to receive a quicker beneficial effect of the drug in the lymphomas than had been necessary in chronic myelogenous leukemia; accordingly, larger doses were given. Twenty-five mg. of Myleran was estimated to be equivalent to 2.5 mg. of triethylene melamine (TEM). In Hodgkin's disease and reticulum cell sarcoma we decided to give 25 mg. of Myleran for 4 doses if the patient had no hematological or chemical abnormality, and then to give no further therapy for a 2-week period. Dosage was continued, based on the blood picture and the therapeutic response. In lymphosarcoma and chronic lymphatic leukemia, due to their apparent increased sensitivity to the alkylating agents, the initial dose was 25 mg. for 2 doses.

Twenty-nine patients with a diagnosis of either chronic lymphatic leukemia, Hodgkin's disease, reticulum cell sarcoma, or lymphosarcoma received Myleran. Six of these patients received an inadequate trial, as judged by failure to produce a significant degree of hematological depression, and 2 are still receiving therapy. Of the 21 remaining patients, 12 were classified as failures, 3 as showing subjective improvement, and 6 as showing both subjective and objective improvement. Four of these latter 6 patients were classified as incomplete remissions. They represented 1 patient with reticulum cell sarcoma who had marked decrease in skin infiltrations, 2 patients with chronic lymphatic leukemia whose hepatosplenomegaly regressed under therapy, and a patient with Hodgkin's disease whose enlarged spleen decreased in size. These objective criteria of improvement, however, were transitory and were measured in weeks. The 2 remaining patients in this group underwent complete remissions (FIGURES 1 and 2).

Discussion

Myleran appeared to be effective in controlling the disease process of these wo patients. In view of the fact that both were in the early stages of their disease when treated with Myleran, it would have been expected that they hould have responded to other alkylating agents also. All such agents may be influenced by the route of administration, dosage schedule, duration of reatment, stage of disease at onset of therapy, and especially the compara-

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* These studies were supported in part by Research Grant CY3215 from the National Cancer Institute, Public Health Service, Bethesda, Md., and in part by institutional grants from the American Cancer Society, the Damon Runyon Fund for Cancer Research, and the asker Foundation, all in New York, N.Y.

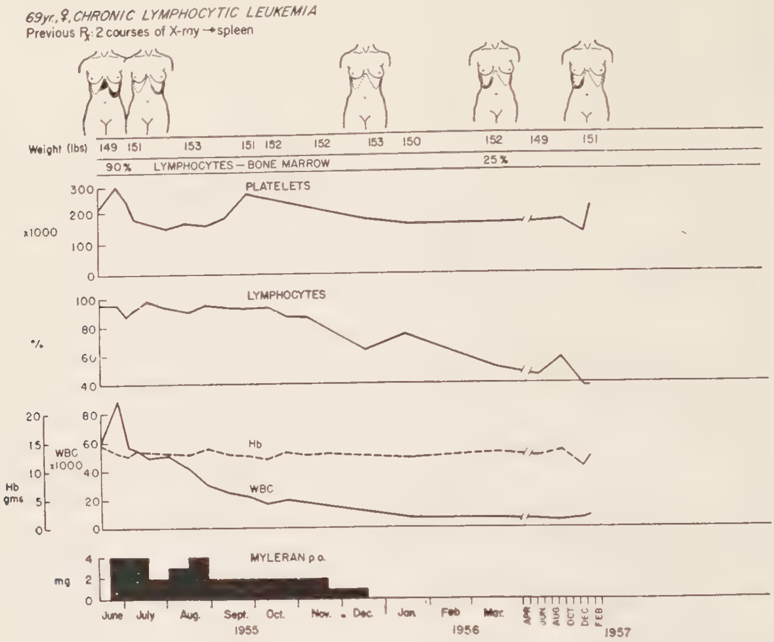

FIGURE 1. Sixty-nine-year-old white female (E. B.) who, in 1949, consulted her family physician with a chief complaint of headaches. A routine blood count revealed an eleva tion of the white blood corpuscles. Physical examination revealed that the spleen was enlarged, and the patient was treated with 800 r to the left upper quadrant, with regression in spleen size. In 1950 she received a second course of splenic irradiation, 600 r. She was first seen at Memorial Center on June 16, 1955. Physical examination showed that the liver was enlarged 7 cm. below the zyphoid process in the midline, and the spleen edge was palpated 6 cm. below the left costal margin. A 1-cm. node was present in the left axilla. A sternal marrow aspiration was consistent with chronic lymphatic leukemia. Her hemoglobin was 14.3 gm.; WBC, 61,000; platelets, 225,000; and the differential showed 95 per cent lymphocytes. She was started on Myleran therapy, 4 mg. daily. In 2 weeks there was diminution in size of both liver and spleen, and the WBC had started to drop. She received a total of 456 mg., which equals 6.7 mg. kg., from June 22 to December 20, 1955. She showed objective improvement on Myleran, as evidenced by a return of sternal marrow to normal, a liver and spleen that no longer were palpable, a decrease in WBC to 11,000, with the lymphocytes in the peripheral blood reduced to 40 per cent. She has received no treatment since December 20, 1955, and no objective evidence of disease is present.

tive rates of speed of the growth of the tumor. As to the effectiveness of Myleran as compared with the chemotherapeutic agents currently employed in the treatment of lymphomas, this would require a comparative study with other polyfunctional alkylating agents to determine any quantitative differences in therapeutic activity and response at various levels of hematological depression.

Summary

Twenty-one patients with either lymphoma or chronic lymphatic leukemi were treated with Myleran, with dosages ranging from 2 to 25 mg. day Twelve of these patients were considered failures in response to treatment, showed subjective improvement, and 6 showed both subjective and objectiv

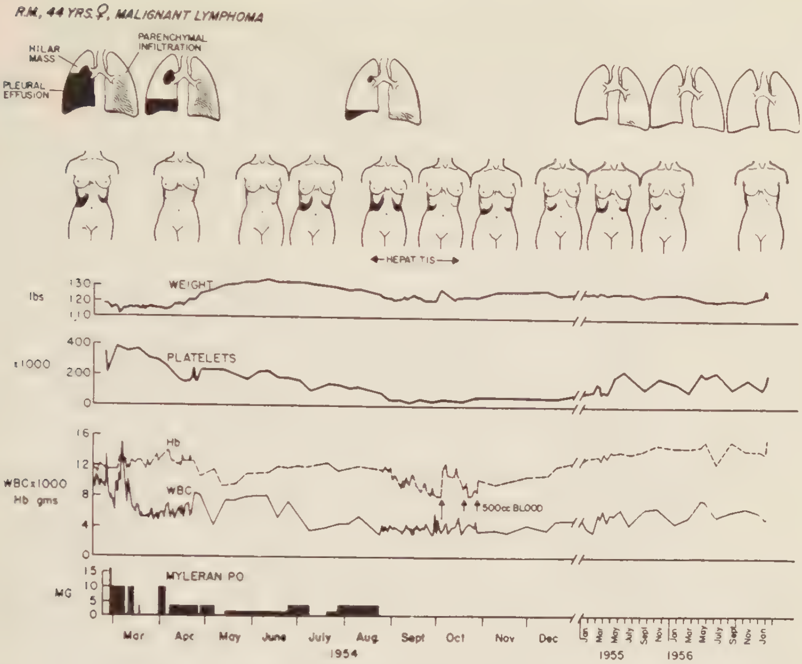

FIGURE 2. Forty-four-year-old female who, in October 1953, had onset of cough. She received penicillin and Terramycin with no improvement. On one occasion she coughed up a small amount of bloody mucus. A chest X ray was taken in November, and she was referred to Memorial Hospital, where she was first seen in January 1954. Positive findings on physical examination consisted of hepatomegaly, 4 fingers below the costal margin and a spleen edge palpated 2 fingers below the costal margin. A chest X ray showed right hilar infiltration, fluid at the right base, and several large patchy areas of metastatic disease in the lower two thirds of the left lung field. A biopsy of a node was diagnosed as malignant lymphoma. The liver was increased in size to 8 cm. below the costal margin, and the spleen 2 cm. down. The patient was febrile daily up to 100°, and her weight was 116 lb. Myleran therapy was begun on February 26, 1954, and continued through August 23, 1954. She received a total dosage of 528 mg. with daily doses varying from 4 to 10 mg. p.o. Her temperature dropped to normal within the first month, and her liver and spleen were no longer palpable after the first 6 weeks of therapy. Her chest films showed progressive improvement in both metastatic infiltration, pleural changes, and decrease in fluid. Three months after the onset of therapy she had gained 18 lb. She developed a leukopenia down to 2.9 thousand and a thrombocytopenia from 381,000 to a low of 27,000. Mild hemorrhagic tendencies appeared, but cleared at cessation of therapy; her platelets, however, continued depressed for about 12 months before returning to normal.

The patient has received no therapy after August 23, 1954, and continues to the present in remission.

improvement. Four of the 6 patients showing both objective and subjective improvement were incomplete remissions and transitory in nature; the remaining 2, however, were complete remissions, and both show no evidence of disease 16 and 32 months, respectively, after treatment.

Reference

1. GALTON, D. A. G. 1956. The use of Myleran and similar agents in chronic leukemias. Advances in Cancer Research. **4:** 73–112. J. P. Greenstein & A. Haddow, Eds.

MYLERAN THERAPY IN BRONCHOGENIC CARCINOMA

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1,4-Dimethanesulfonyloxybutane (Myleran) has been reported to be effective in chronic myelogenous leukemia, but lacking antitumor activity in other types of neoplastic disease.[1-5] More recently, Sykes *et al.*[6] observed clinical response in chronic lymphatic leukemia and malignant lymphoma. The current study was designed to investigate the effects of Myleran in bronchogenic carcinoma, using dosage schedules comparable in bone marrow depressant activity to that resulting from intravenous nitrogen mustard (HN2).

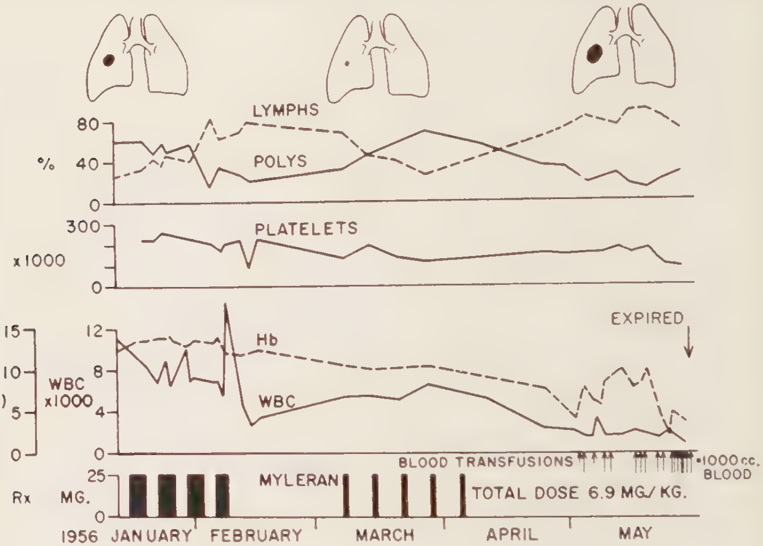

FIGURE 1. Case 1.

Various Myleran dosage schedules were explored to establish the dosage given over 2 to 4 days, that would result in transient leukopenia. The compound was given once daily by mouth, 1 hour before breakfast, in multiple of 25 mg., in dosages ranging from 0.5 mg./kg. daily for 4 days to 2.0 mg./ kg./day for 2 days. The majority of patients received 1.0 mg./kg./day fo 4 days (approximately 50 mg./day for 4 days).

Of twenty-four patients who received a full course of Myleran therapy 7 died of their disease, without leukopenia, before the tenth day, and wer considered inadequate trials (TABLE 1). The remaining 17 patients evaluated for antitumor activity included 6 (35 per cent) with objective evidenc of tumor regression. This was manifested by decrease in size of measurabl pulmonary lesions on serial roentgenogram (5 patients), decrease in venou

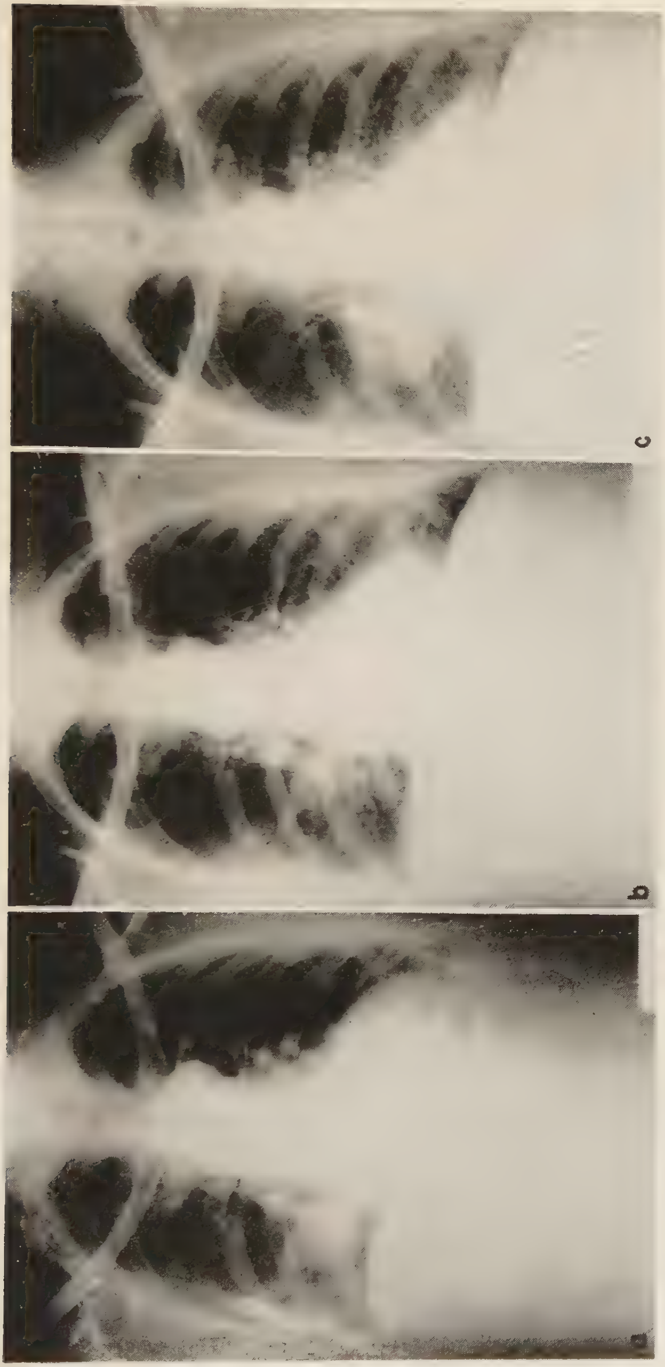

FIGURE 2. Case 2: (a) before therapy, (b) 58 days after therapy, and (c) 120 days after therapy.

Table 1
Myleran Therapy in Bronchogenic Carcinoma

Case No.	Age Sex	No. days treated 1st Course (1) 2nd Course (2)	Maximum daily dose (mg.)	Total dose mg.	Total dose mg./kg.	Hematological toxicity: No. days* onset ↓ WBC	Greatest ↓ WBC	No. days* return to normal	Hb ↓ (gm.)	Plat. count depression†	Response	Duration response (mo.)	Remarks
1	60 M	(1) 15 (2) 5	25 25	350 125	5.2 1.7	30 28	2,700 800	54 —	3.0 7.0	2 2	Decrease RML lesion	2½	Died 82nd day after 2nd course ℞ with pancytopenia
2	62 M	(1) 8 (2) 4	50 25	300 100	4.8 1.8	17 24	1,000 2,000	35 41	4.0 6.0	2 2	Decrease RUL lesion, ↓ V.P., improvement in neurological signs	1½	Died 41st day after 2nd course ℞
3	45 M	8	50	350	4.2	16	2,900	11	3.0	1	Decrease mediastinal widening	3+	Other form therapy 7 months later
4	66 M	4	50	200	4.0	17	1,500	36	2.0	0	Decrease hilar lesion, improvement in neurological signs	2	Died 73rd day
5	39 M	(1) 3 (2) 2	75 75	200 150	3.5 2.9	18 16	2,800 2,200	32 44	0 1.0	1 1	↓ V.P. 350 to 260	6 weeks	Died 44th day after 2nd course ℞
6	39 M	4	50	200	3.2	18	3,500	24	1.5	0	↓ Lesion LUL	2+	Died 70th day
7	45 M	13	25	375	6.0	27	2,300	39	2.6	0	No response§	—	Died 71st day
8	38 M	10	50	325	5.8	17	1,600	52	3.0	1	No response	—	Died 52nd day
9	46 M	8	50	300	4.9	27	3,000	38	0	1	No response	—	Died 122nd day
10	38 M	(1) 11 (2) 4	50 50	350 200	5.9 3.2	25 12	3,500 1,500	39 —	0 5.0	1 3	No response	—	Died 188th day after 2nd course ℞ with pancytopenia

11	47 M	11	25	375	4.4	—	—	—	—	—	No response§	—	Died 19th day
12	64 M	8	50	300	5.5	14	500	—	3.0	2	No response§	—	Died 263rd day with pancytopenia
13	63 M	3	75	200	2.7	12	800	—‡	4.0	2	No response§	—	Died 25th day
14	68 M	4	50	200	4.0	15	1,200	—‡	0	0	No response	—	Died 28th day
15	64 M	4	50	200	4.6	14	1,200	—‡	2.0	2	No response	—	Died 20th day
16	60 M	2	100	200	4.0	16	2,900	—‡	2.0	2	No response	—	Died 17th day
17	52 M	4	50	200	4.0	11	500	—‡	—	—	No reponse	—	Died 13th day
18	48 M	2	100	200	2.2	—	—	—	—	—	Inadequate trial	—	Died 3rd day
19	40 M	4	50	200	3.6	—	—	—	—	—	Inadequate trial	—	Died 10th day
20	64 M	3	50	150	2.7	—	—	—	—	—	Inadequate trial	—	Died 3rd day
21	67 M	3	50	150	2.0	—	—	—	—	—	Inadequate trial	—	Died 3rd day
22	63 M	3	50	150	3.0	—	—	—	—	—	Inadequate trial	—	Died 3rd day
23	62 M	2	100	200	3.8	—	—	—	—	—	Inadequate trial	—	Died 10th day
24	65 M	4	50	200	4.0	—	—	—	—	—	Inadequate trial	—	Died 6th day

* Number of days after *onset* of therapy.

† Platelet count depression: 0 = >150,000; 1 = 100,000 to 150,000; 2 = 50,000 to 100,000; 3 = 25,000 to 50,000.

‡ Died before total effects on hemogram could be evaluated.

§ No measurable disease.

pressure in cases with superior vena caval obstruction (2 patients), and improvement in abnormal neurological signs in cases with central nervous system metastases (2 patients). The duration of improvement was 2 weeks to 3+ months. Seven patients showed no objective evidence of improvement and were considered therapeutic failures. Four patients without readily measurable lesions, whose therapy was difficult to evaluate, were included as failures.

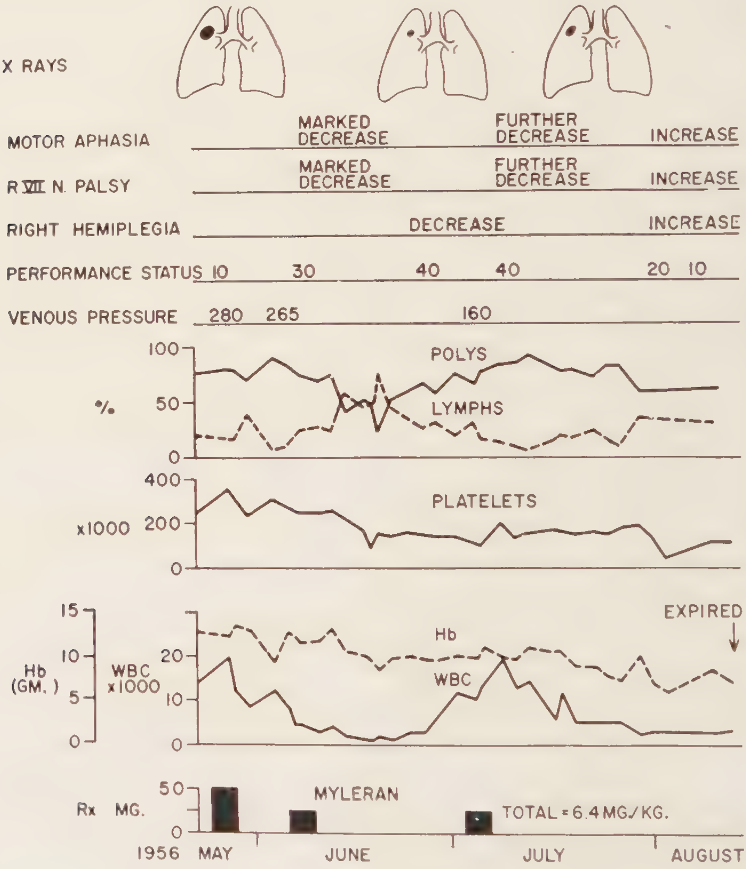

FIGURE 3. Case 2.

The following case reports illustrate the objective tumor regression observed with Myleran therapy:

Case 1. A 60-year-old white man was found to have a tumor of the right middle lobe at thoracotomy in December 1955. The histological diagnosis was oat cell carcinoma. Myleran therapy was begun in multiples of 25 mg./day to a total dosage of 5.2 mg./kg. over a period of 24 days. After a transient leukopenia abated, maintenance therapy was given at weekly intervals (FIGURE 1). Serial roentgenograms (FIGURES 2a and 2b) revealed

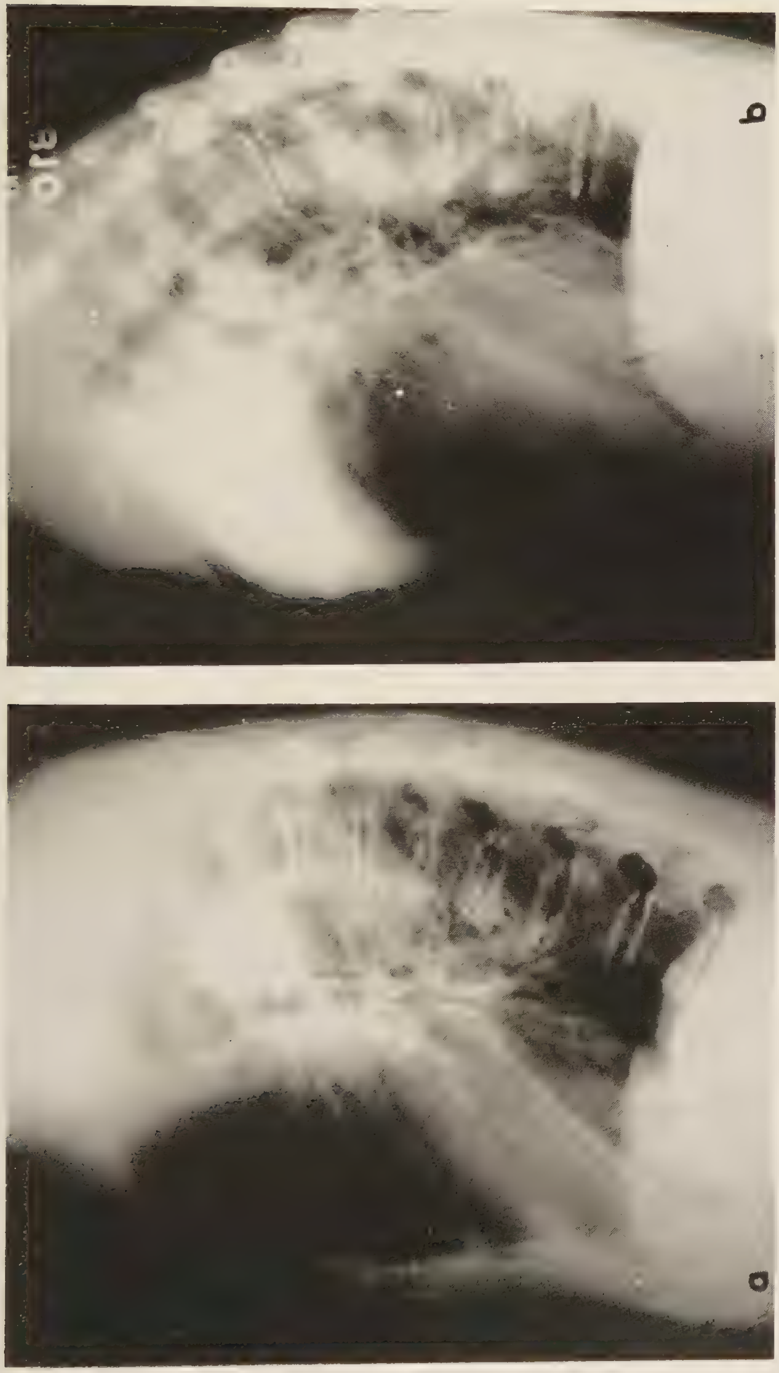

FIGURE 4. Case 2: (a) before therapy and (b) after therapy.

a gradual decrease in the size of a discrete right middle lobe lesion, with the greatest reduction occurring 58 days after the onset of therapy. Regrowth of the same lesion was apparent by the 72nd day and was progressive (FIGURE 2c) until the patient died with generalized metastases on the 145th day. Progressive pancytopenia developed following maintenance therapy, and was associated with petechiae, epistaxis, and gastrointestinal bleeding, the latter probably being related to the mechanism of death of the patient.

Case 2. A 62-year-old white man was found to have bronchogenic carcinoma in April 1956. The patient exhibited a discrete right upper lobe lesion, superior vena caval syndrome with elevated venous pressure, and central nervous system signs, including motor aphasia, right facial palsy, and right hemiplegia which were regarded as due to cerebral metastases (FIGURE 3). Myleran therapy was given in a total dosage of 4.8 mg. kg. over a period of 15 days. Following treatment, there was progressive decrease in size of the right upper lobe lesion (FIGURE 4), reduction in venous pressure from 280 mm. H_2O to 160 mm. H_2O, and improvement in motor aphasia and regression of abnormal neurological signs. After the hematological effects had abated, a second course of Myleran was given in a total dosage of 1.8 mg. kg. Clinical benefit persisted for 6 weeks, following which there was evidence of increased tumor growth, with progressive central nervous system involvement and death on the 80th day after the onset of therapy. A moder-

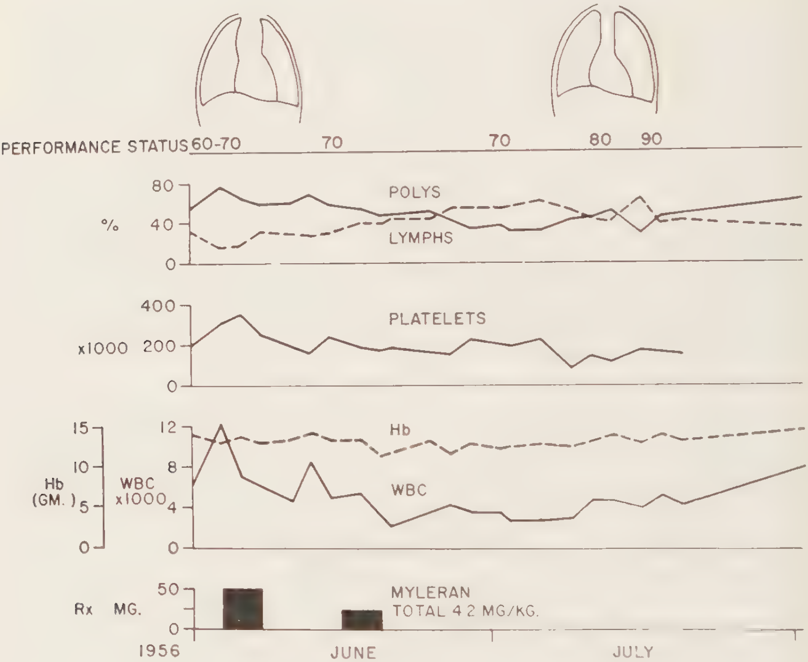

FIGURE 5. Case 3.

ate pancytopenia developed after the second course of Myleran and persisted until the patient's death.

Case 3. A 45-year-old white man was found to have carcinoma of the right upper lobe at bronchoscopy in May 1956. The only objective parameter for drug response was a widening of the right superior mediastinum. One course of Myleran therapy was given in June 1956 in a total dosage of 4.2 mg./kg. of body weight (FIGURE 5). There was progressive decrease in the size of the mediastinal mass within 2 weeks of the onset of therapy until the patient was lost to follow-up 2 months later. When he returned to the hospital in January 1957, there was evidence of increased tumor growth.

All patients evaluated for antitumor activity developed varying degrees of leukopenia, except one (Case 11). In 10 patients, leukopenia was of moderate degree, occurring between the 11th and the 30th day after the onset of therapy, and was improved by the 24th to the 54th day. A slight and transient decrease in hemoglobin and platelet counts occurred in most of these patients. Five patients died before the *total* effects on the hemogram could be evaluated. One patient developed a pancytopenia (Case 12) of moderate degree, persisting until he succumbed to his disease 9 months after Myleran therapy. Four patients received a second course of Myleran therapy with the development of varying degrees of pancytopenia. Two patients recovered from the hematological depression on the 41st and 44th day following therapy (Cases 2 and 5), while 2 continued to show pancytopenia until death from the neoplastic process on the 82nd and 188th day, respectively (Cases 1 and 10). Otherwise, in the dosage used the compound was well tolerated by all patients and was notable for its lack of untoward gastrointestinal symptoms.

Conclusion

In this study, Myleran in doses of 1.0 mg./kg./day for 4 days produced bone marrow depression comparable to that due to the usual dose of intravenous nitrogen mustard (that is, 0.1 mg./kg./day for 4 days). Objective evidence of tumor regression was noted in 6 of 17 patients under study. The results are considered similar to those seen with nitrogen mustard. When courses of Myleran therapy were repeated in this dosage, there was persistent pancytopenia in 2 patients.

References

1. Haddow, A. & G. M. Timmis. 1953. Myleran in chronic myelogenous leukemia. Chemical constitution and biologic action. Lancet. **264:** 207.
2. Galton, D. A. G. 1953. Myleran in chronic myeloid leukemia. Lancet. **264:** 208.
3. Hyman, G. A. & A. Gellhorn. 1956. Myleran therapy in malignant neoplastic disease. J. Am. Med. Assoc. **161:** 844–849.
4. Petrakis, L., H. D. Bierman, K. H. Kelly, L. P. White & M. B. Shimkin. 1954. The effect of Myleran upon leukemia. Cancer. **7:** 383–390.
5. Frost, J. W. & C. B. Jackson. 1956. Myleran in the treatment of chronic granulocytic leukemia. J. Am. Med. Assoc. **161:** 54–56.
6. Sykes, M. P., F. S. Philips & D. A. Karnofsky. 1956. Comparative therapeutic activity of the nitrogen mustards and allied compounds. Med. Clin. N. Am. **40:** 837–856.

EXPERIMENTAL AND CLINICAL EXPERIENCE WITH THIO-TEPA IN LEUKEMIA AND INOPERABLE CANCER

By Harry Shay and David C. H. Sun

Fels Research Institute and Temple University Medical Center, Temple University School of Medicine, Philadelphia, Pa.

In 1952 Personeus and his associates,[1] investigating the effect of a group of ethylenimine derivatives on a transplantable metastasizing mammary adenocarcinoma in inbred Hooded rats, found triethylene thiophosphoramide, Thio-TEPA (or Tespa, as Lederle Laboratories, Pearl River, N. Y., are now labeling the compound), to be among the stable compounds most active in inhibiting the development of secondary metastatic foci in the lung.

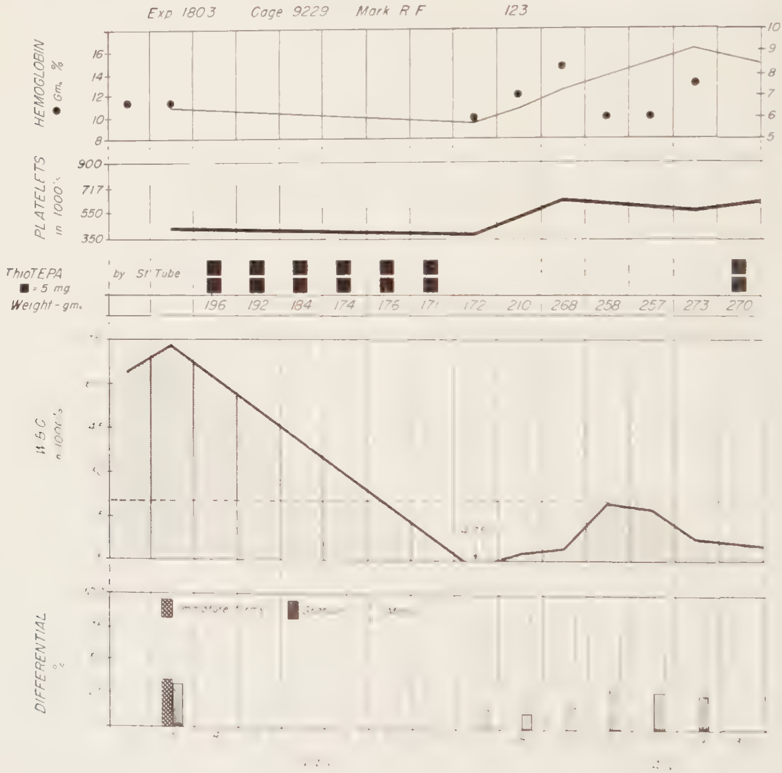

FIGURE 1. Hematological response in a chloroleukemic rat following gastric instillation of Thio-TEPA.

Sparks *et al.*[2] showed that, when administered intraperitoneally to Sherman rats bearing a transplanted chloroleukemia originally induced in a Wistar rat in our laboratory,[3] Thio-TEPA had a profound effect on this leukemia. We[4, 5] were able readily to reproduce their results with Thio-TEPA administered to leukemic Wistar rats of our own colony and to demonstrate further that the agent was as effective when administered by stomach catheter as when given intraperitoneally, the latter being the portal of administration used by Sparks and her associates.

FIGURE 1 illustrates the usual response obtained in the chloroleukemic rat following gastric instillation of Thio-TEPA. This response was a rapid hematological and clinical remission that in some rats persisted for very long periods after only 1 course of 6 daily doses of 10 mg. each.

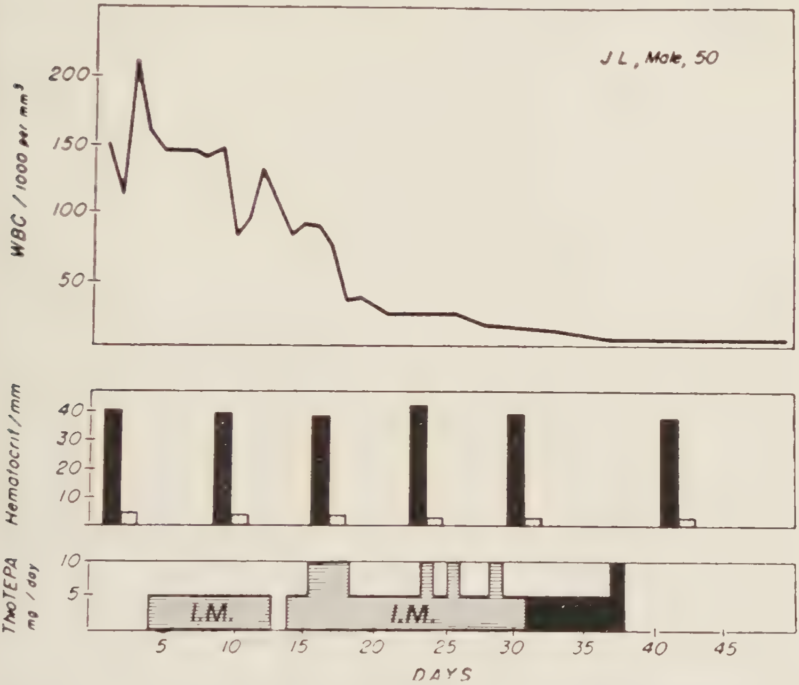

FIGURE 2. Hematological course of patient J. L. during treatment with intramuscular and intravenous triethylene thiophosphoramide. The solid areas indicate packed red blood cells; the dotted areas, packed white blood cells.

These encouraging results obtained with Thio-TEPA in leukemic rats, coupled with what appeared to be an action of lesser intensity upon the bone marrow and peripheral blood of normal rats, prompted a cautious trial of Thio-TEPA in human chronic leukemia.[4]

The first patient treated was suffering from active chronic lymphocytic leukemia. J. L., a white male, age 50, presented chief complaints of having had "lump on back of neck" for 1 year, and of weakness, fatigability, and abdominal discomfort for 3 months prior to admission. Physical examination disclosed generalized lymphadenopathy with masses of nodes in the neck and both axillary regions, as well as an epigastric mass extending 5 cm. below the umbilicus. The latter was interpreted to be a mass of abdominal lymph nodes. Bone marrow biopsy revealed a very highly cellular marrow with marked infiltration by lymphocytic elements. Thio-TEPA was started in October 1952, at which time the total leukocytic count was 160,000 per cu. mm., hemoglobin 12.7 gm., and platelets, 149,000 per cu. mm. FIGURE 2 shows the patient's hematological response over the first 45 days of treatment.

This patient lived near Pittsburgh, Pa., and therapy with Thio-TEPA was continued through his local physician after his discharge from the hospital. Six months after starting treatment with the drug, he had gained 13 lb. in weight, was eating well, and had returned to work for the preceding 2 months. His abdominal mass was no longer palpable, and his axillary and neck masses had receded to discrete pea-size nodes.

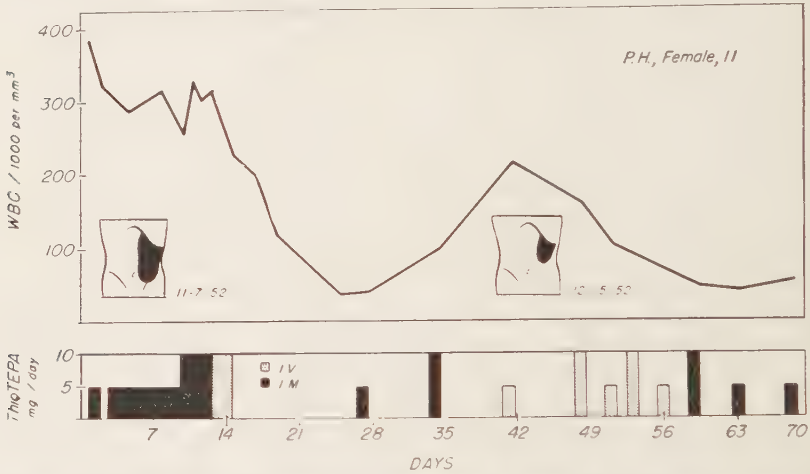

FIGURE 3. Hematological course and spleen size in patient P. H., chronic (subacute) granulocytic leukemia treated with intravenous and intramuscular Thio-TEPA.

The patient was maintained on intermittent therapy until June 1954, a period of 20 months, when Thio-TEPA was discontinued because of a low platelet count. His physician then treated him with cortisone. He improved for approximately 2 months, with a return of his platelets to 160,000 per cu. mm., following which his leukocyte count again began to rise, his lymph nodes increased in size, and an abdominal mass was again palpable. Sweating and weakness increased and, early in October 1954, the patient began to have increasing abdominal colicky pain. He was admitted to a local hospital near his home with a diagnosis of partial intestinal obstruction. He became progressively weaker and died November 2, 1954, 2 years and 1 month after initial Thio-TEPA therapy.

The first case of myelogenous leukemia to be treated with Thio-TEPA was patient P. H., an 11-year-old white female, in September 1952. This child, a subacute myelogenous leukemia with the tip of the spleen reaching 15 cm. below the left costal margin and showing an initial leukocyte count of 360,000 per cu. mm., went into and was maintained in good remission with Thio-

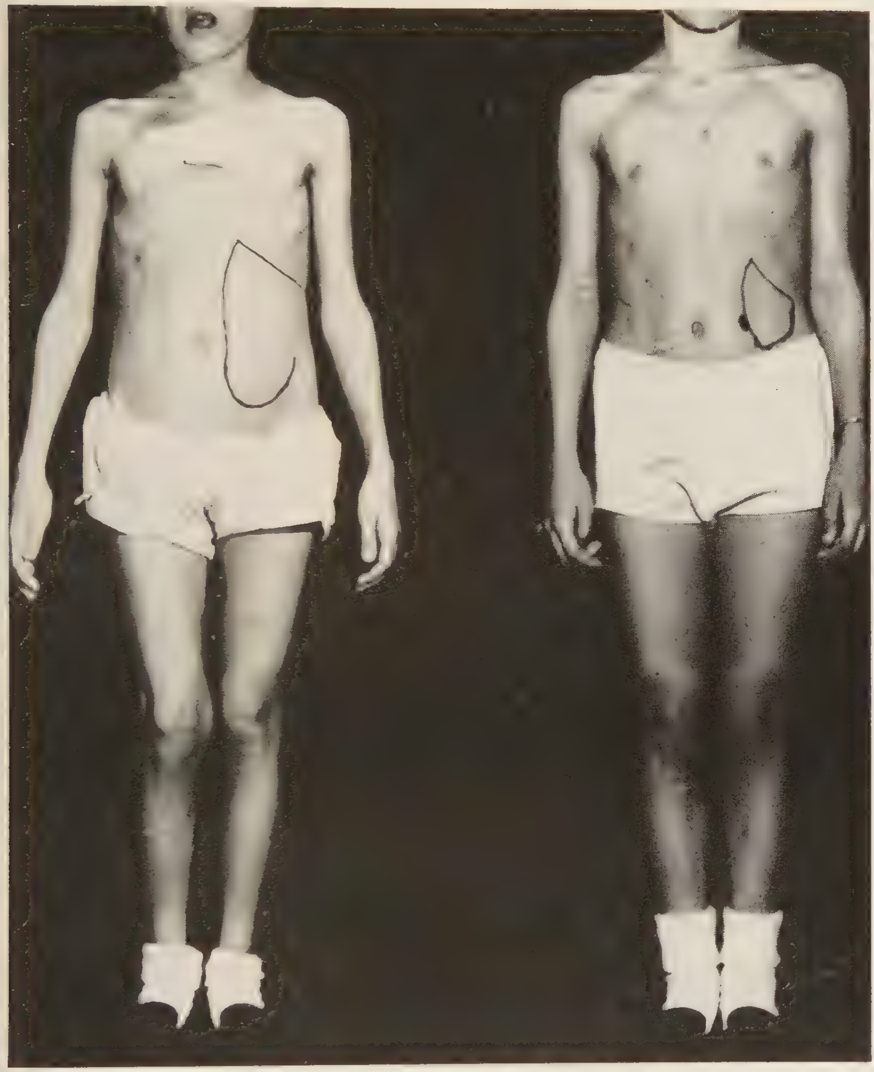

FIGURE 4. Reduction in the size of the spleen and improvement in nutritional status in patient P. H. after 6 weeks of Thio-TEPA therapy.

TEPA for nearly 2 years (FIGURES 3 and 4). Her leukemia then became refractory to Thio-TEPA, after which she was maintained in remission with Myleran (1,4-dimethanesulfonyloxybutane) for another year before she became refractive to this drug as well. She was not under our care at the time; it was therefore not possible to learn whether she might not then have responded to Thio-TEPA once more. We learned later that she was placed on urethan, but her course continued rapidly downhill and she died in November 1955. Most of the details on these 2 patients have been reported previously.[4]

In the case of patient S. S., a 53-year-old white female, a diagnosis of chronic myelogenous leukemia was made from bone marrow biopsy at Lenox Hill Hospital, New York, N. Y., in December 1952. She was referred to us for treatment in March 1954, with a chief complaint of marked weakness. Her blood count then was, hemoglobin 10.9 gm., RBC. 3.5 million per cu. mm., leukocytes 190,000 per cu. mm., with the differential showing 97 per cent cells of the myelocytic series of which 3 were myeloblasts, 6 promyelocytes, 26 myelocytes, and 21 metamyelocytes. The spleen was palpable 5 cm. below the left costal margin. She was started on oral Thio-TEPA, with rapid clinical and hematological improvement. The drug administration and hematological responses are shown in FIGURE 5.

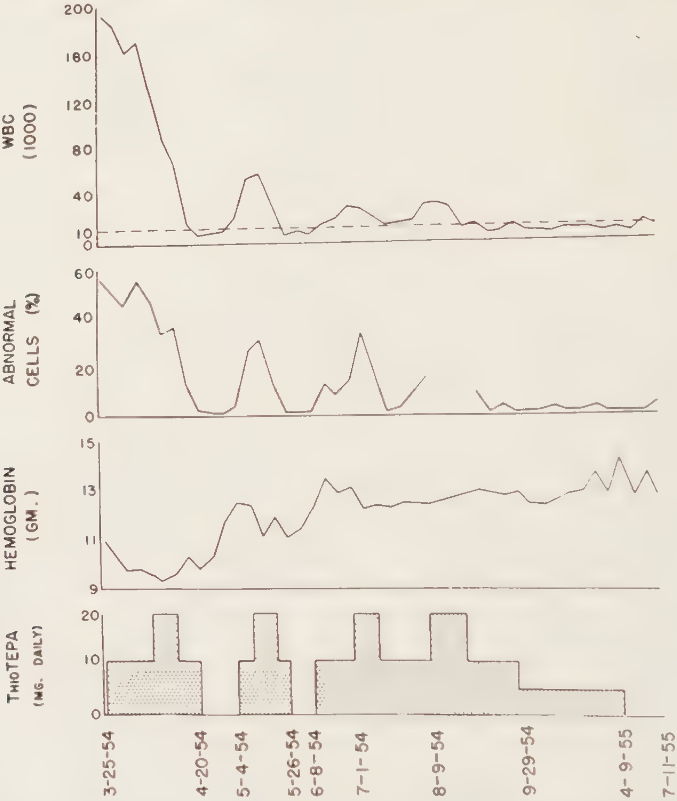

FIGURE 5. Hematological course in patient S. S., chronic myelogenous leukemia treated with oral Thio TEPA.

Because of our previous experience with the action of Thio-TEPA in our chloroleukemia in rats,[5] in which 1 course of the drug brought about a remission that lasted in some rats for as long as 1 year, a remission that, on a comparative time schedule in the life span of man, would be tantamount to a cure, we were prompted to attempt treatment in this patient not only to bring about a remission, but a peripheral blood and bone marrow picture that might be indistinguishable from the normal. This was based on the opinion[6] that any hope for eradicating leukemia in any patient must include a return to a normal blood and bone marrow picture, a status that should be sought even though the opinion of many hematologists currently holds that reduction in cell count alone is not a vital consideration in the treatment of the disease. We are glad to find that Galton *et al.*, elsewhere in this monograph (p. 967), emphasize the desirability of approaching a normal peripheral white blood count in maintaining remission in chronic myelogenous leukemia. The normal peripheral blood and bone marrow picture was achieved in this patient, but only intermittently, even though treatment with Thio-TEPA was continuous.

She was maintained in satisfactory clinical and hematological remission until July 1956, when her leukemia became refractory to the drug after 28 months of therapy. She was then started on Myleran, to which her leukemia responded well. In October 1956, she had an upper gastrointestinal hemorrhage, attributed at the time to a hiatal hernia, and was admitted to Mt. Sinai Hospital, New York, N. Y. She was given 7 blood transfusions of 500 cc. each and made a good recovery from her gastrointestinal bleeding. She has since been maintained in satisfactory remission with Myleran for a total of 8 months under the care of L. R. Wasserman of Mt. Sinai Hospital.

Our results in the last two cases, in their initial and prolonged response to Thio-TEPA with final refractoriness to the drug followed by good and sustained remissions after Myleran, represent perhaps different points of attack of the two drugs and suggest the possibility of shifting from one to the other in the prolonged management of chronic myelogenous leukemia. We have not yet had the opportunity to test whether refractoriness to Thio-TEPA is lost after prolonged treatment with Myleran, nor have we been able to test whether Thio-TEPA would prove effective in a patient who has been treated initially with Myleran and has become refractive to it.

Perhaps alternate use of the two drugs, as the leukemia becomes refractory to one or the other, or after a period of treatment with one or the other drug before refractoriness occurs, might permit an actual prolongation of life in these patients. We have been at a loss to understand the enthusiasm shown in testing Thio-TEPA in inoperable cancer, and the complete lack of interest in its use in chronic leukemia, for it is in chronic myelogenous and chronic lymphatic leukemia that we have found it most useful.[4, 7]

Early in 1953,[4] shortly after we became aware of the effectiveness of Thio-TEPA in chronic leukemia, we tried the drug in patients with inoperable carcinoma. The first patient treated was a young Negro woman, J. S., age 30, who had recurrence and metastasis after a right radical mastectomy in February 1952 for adenocarcinoma of the breast. In spite of bilateral salpingo-oöphorectomy, adequate roentgen-ray and testosterone therapy, local recurrence and spread of the disease continued and involved the lymph nodes in the axilla of the opposite side, as well as skin seeding, one area of

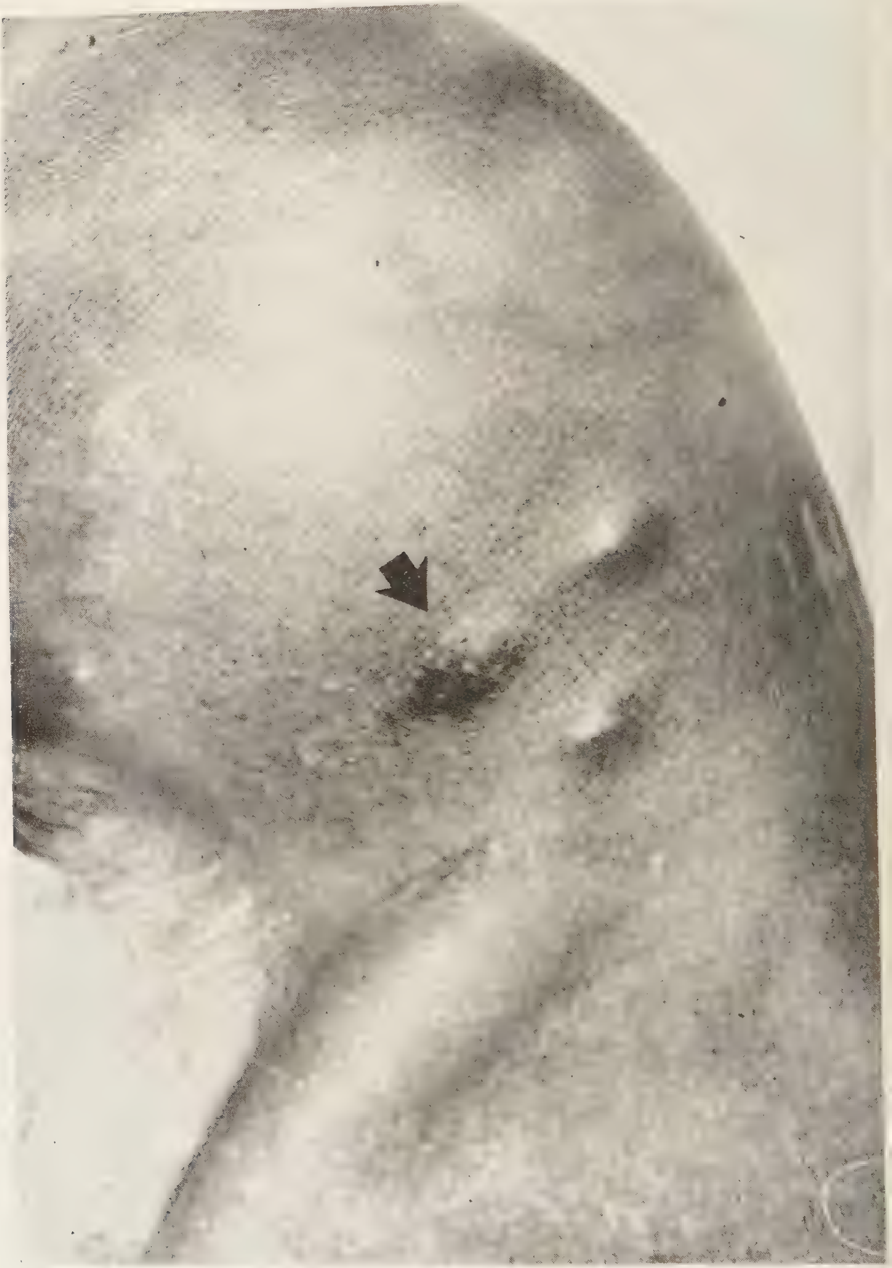

FIGURE 6. Subcutaneous metastatic tumor nodules on the right shoulder of patient J. S. The scar on the top of the shoulder was one of the 4 nodules removed for histological examination. The nodule injected locally with Thio-TEPA showed black pigmentation of the skin overlying the nodule (arrow). This was removed subsequently and serial sections were made.

which is shown in FIGURE 6. She responded most satisfactorily, objectively and clinically, to intravenous Thio-TEPA, our usual and recommended avenue for treatment of inoperable cancer with this drug. However, the areas of skin seeding on her shoulder failed to undergo any discernible change. One of these was removed surgically and histological section showed metastatic carcinoma (FIGURE 7). Unorthodox though it may be, we then treated the second nodule by local injection of Thio-TEPA. There was flattening of the nodule and increased pigmentation of the skin overlying the nodule. The area that had contained the nodule was then removed, and 170 serial sections of the resected specimen were made. Histological examination of these sections failed to show any cancer cells (FIGURE 8).

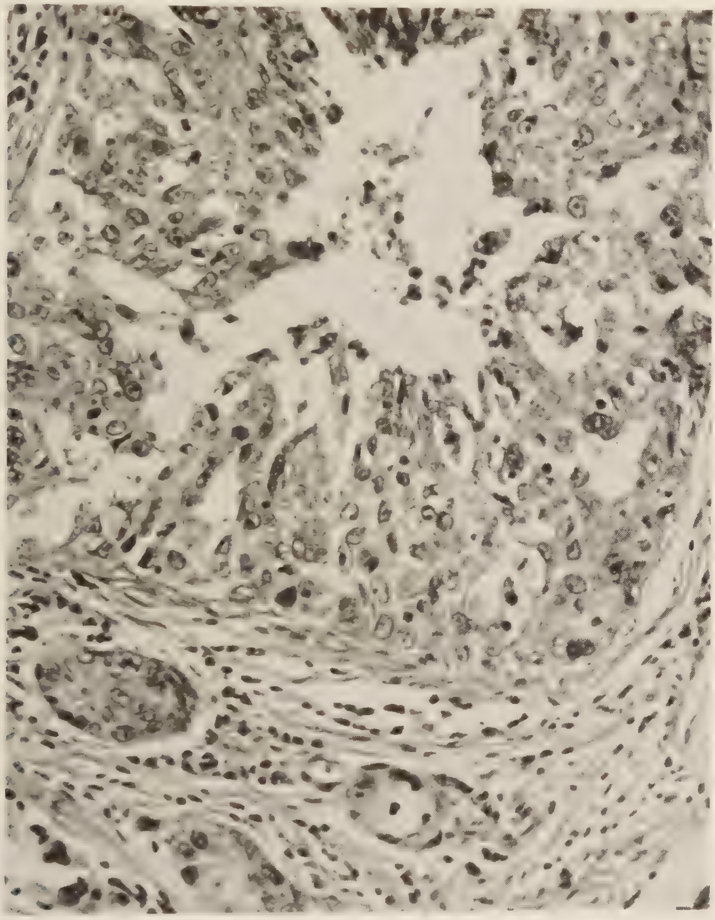

FIGURE 7. Pretreatment biopsy of subcutaneous tumor nodule: adenocarcinoma, metastatic from the breast. Hematoxylin-eosin stain. × 600.

In a report of 47 patients[8] with various types of inoperable, far advanced metastatic cancer treated with Thio-TEPA, the most encouraging results, both objectively and subjectively, were seen in patients with carcinoma of the breast and carcinoma of the ovary. However, improvement was limited, and lesions that at first responded, sooner or later became resistant, and new metastases developed in spite of continued treatment. In some cases no initial response was obtained. Jane Wright *et al.*, elsewhere in these pages (p. 937) report essentially parallel results in their study of the treatment of 93 cases of inoperable carcinoma.

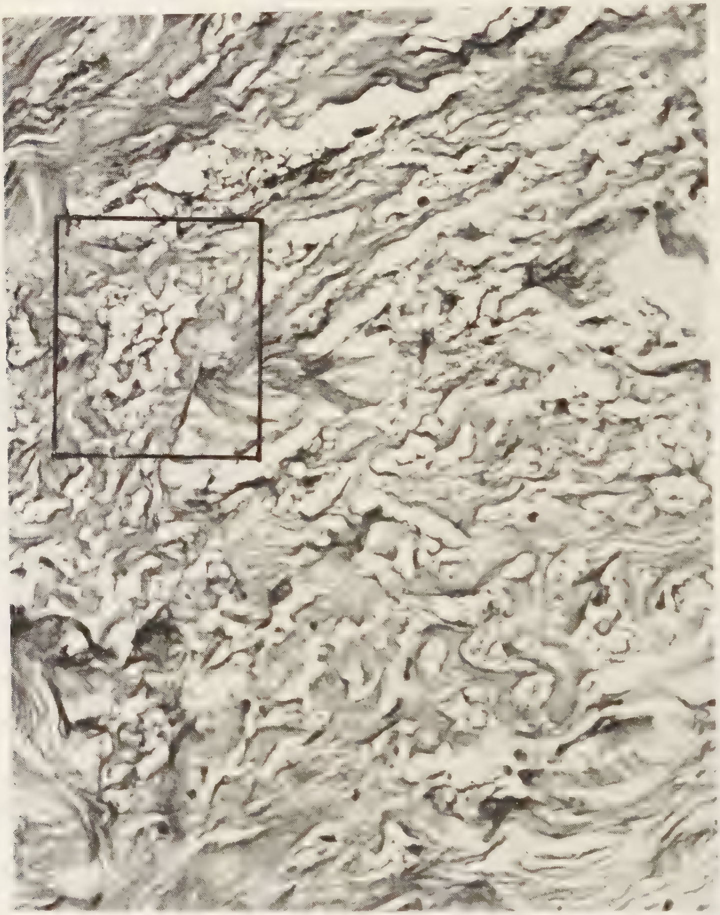

FIGURE 8. Adjacent tumor nodule, after local injection of Thio TEPA. The residual stromal pattern failed to show any tumor cells. Hematoxylin-eosin stain. × 600.

A rather high incidence of serious delayed bone marrow depressions is reported elsewhere in this monograph by Ultmann *et al.* (p. 1007) and by Olson (p. 1018) in describing their series of inoperable cancer treated with Thio TEPA. This finding has supported our earlier caution in the use of this drug that there should be no standard dosage for all patients, and that the dosage level on a body-weight basis is not reliable. Treatment should be "individualized" for each patient, and an effort to "titrate" the response of the bone marrow and peripheral blood to Thio-TEPA is imperative. Once we followed the dosage schedule we recommended,[5] we did not encounter any serious bone marrow depression. Wright *et al.*, in treating their patients on an individualized basis, reported no serious toxic effects in their series.

The fact that normal bone marrow in different patients may exhibit different degrees of sensitivity led us to conclude that no initial dose should ever be larger than 10 mg., and should not be that large if the total white cell count is less than 10,000 per cu. mm. Furthermore, each additional dose should be determined by the level of the white cell count in terms of 1 mg. per 1000 WBC. A platelet count should be done once a week. The size of any one dose and the intervals between the injections are guided by the hematological response. With this schedule, the daily dose is reduced to 0.5 mg. per 1000 WBC when the count falls between 5000 and 4000, and the drug is discontinued when the white cell count reaches 4000, the platelet count goes below 150,000 per cu. mm., or when a sudden drop in red cell count or hemoglobin occurs. Such a schedule, we believe, would in a large measure eliminate the severe delayed bone marrow depressions.

We should like to emphasize again that, in patients with roentgenographic or other evidence of bony metastasis or in patients with infection, Thio-TEPA should be administered with caution. In addition, in patients who have been treated recently with other radiomimetic agents, Thio-TEPA should be used cautiously. It is our impression that an additional stress reaction, such as a surgical operation, a trauma, or a systemic infection, may precipitate a serious bone marrow depression during treatment with Thio-TEPA. If the bone marrow is hypocellular, the drug should be discontinued temporarily.

We are not suggesting that Thio-TEPA is any real answer to the problem of treatment of inoperable cancer, but we continue to subscribe to the opinion expressed originally, that the drug is an agent worthy of trial in recurrent and metastatic cancer of breast and ovary and merits addition to the small group of chemotherapeutic agents that have had some usefulness in the palliation of inoperable neoplastic disease.

References

1. Personeus, G., S. L. Halliday, D. McKenzie & J. H. Williams. 1952. Effect of a series of ethylenimine derivatives against metastasizing mammary adenocarcinoma of the rat. Proc. Soc. Exptl. Biol. Med. **81**: 614–616.
2. Sparks, S. J., M. L. Stevens, M. J. Landes, S. L. Halliday, D. McKenzie & J. H. Williams. 1953. The effect of a series of ethylenimine derivatives on myeloid chloroleukemia in the rat. Blood. **8**: 655–660.
3. Shay, H., M. Gruenstein, C. Harris & L. Glazer. 1952. Transfer of myelogenous leukemia induced by gastric instillation of methylcholanthrene. Blood. **7**: 613–622.
4. Shay, H., C. Zarafonetis, N. Smith, I. Woldow & D. C. H. Sun. 1953. Treatment of leukemia with triethylene thiophosphoramide (Thio-TEPA). Preliminary results in experimental and clinical leukemia. A.M.A. Arch. Internal Med. **92**: 628–645.
5. Shay, H., M. Gruenstein & C. Harris. 1954. The effect of triethylene thiophosphoramide (Thio-TEPA) in the treatment of chronic myelocytic chloroleukemia in the rat. J. Natl. Cancer Inst. **15**: 463–481.

6. Shay, H., M. Gruenstein & C. Harris. 1955. Experimental leukemias in the rat. Their development and transfer and some applications to the human problem. Acta Haematol. **14**: 337–353.
7. Zarafonetis, C. J. D., H. Shay & D. C. H. Sun. 1955. Triethylene thiophosphoramide in the treatment of chronic leukemia. Cancer. **8**: 512–522.
8. Shay, H. & D. C. H. Sun. 1955. Clinical studies of triethylene thiophosphoramide in the treatment of inoperable cancer. Cancer. **8**: 498–511.

PALLIATION OF CANCER IN HUMAN PATIENTS BY MAINTENANCE THERAPY WITH *NN'N''*-TRIETHYLENE THIOPHOSPHORAMIDE AND *N*-(3-OXAPENTAMETHYLENE)-*N'N''*-DIETHYLENE PHOSPHORAMIDE*

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Since World War II a number of polyfunctioning alkylating agents, generally known as the nitrogen mustard drugs, have been used clinically. Best known are methyl-bis(β-chlorethyl)amine hydrochloride or HN2, triethylene melamine or TEM, and triethylene phosphoramide or TEPA. Their chief usefulness has been in the treatment of the lymphomata. Unfortunately, none appear to be equally effective against the epithelial or "solid" tumors. Although TEM can be given orally, the vesicant nature of these agents generally limits the possible routes of therapy.

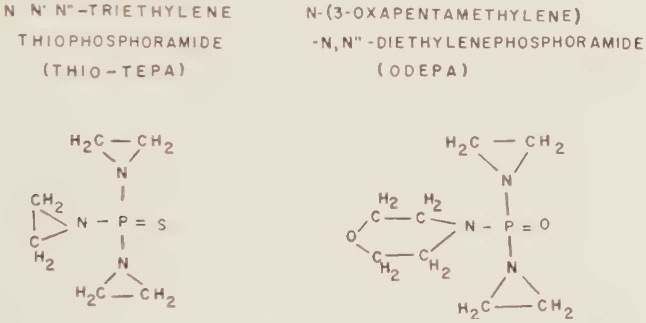
N N' N"-TRIETHYLENE THIOPHOSPHORAMIDE (THIO-TEPA)
N-(3-OXAPENTAMETHYLENE) -N,N"-DIETHYLENEPHOSPHORAMIDE (ODEPA)
H_2C
CH_2
N
P
S
O
C
H_2

FIGURE 1

Beginning in 1953, two newer phosphoramides, *NN'N''*-triethylene thiophosphoramide or Thio-TEPA and *N*-(3-oxapentamethylene)-*N'N''*-diethylene phosphoramide or ODEPA† (FIGURE 1) have been used by us in four clinics‡ for treatment of widespread cancer. These drugs are characterized by: (1) unusual effectiveness against certain solid tumors in human patients; (2) ease of administration; (3) a high therapeutic ratio; (4) toleration by the patient for prolonged or indefinite periods of time; and (5) limitation of dose by hematopoietic reserve of the patient.

A total of 486 patients with far-advanced cancer who were treated is reported. This includes 13 patients with lymphosarcoma or Hodgkin's

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† Supplied through the courtesy of James M. Ruegsegger.

‡ Cancer Chemotherapy Clinic, Garfield Memorial Hospital, Washington, D. C.; Cancer Chemotherapy Clinic, St. Elizabeth's Hospital, Washington, D. C.; Cancer Chemotherapy Clinic, Prince George's County Hospital, Cheverly, Md.; and Cancer Clinic, George Washington University Hospital, Washington, D. C.

disease. Ten individuals had 2 primary tumors; 4 patients had both mammary and large-bowel cancer; 2 patients had bronchogenic cancer and "cured" breast cancer; 2 patients had uncontrolled mammary and cervical cancer; 1 patient had "cured" cancer of the tonsil and active ovarian cancer; and 1 patient had "cured" mammary cancer and active ovarian cancer.

Diagnosis was made from surgical specimens or biopsies in all but a few cases, in which the diagnosis rested on the isolation of cancer cells from serous cavity effusions. All of the patients had had surgery and or X-ray therapy, except for 19 patients who were considered unsuitable for such treatment or who had refused conventional therapy. In 24 cases chemotherapy was instituted at the time of, or immediately after, the original surgery for incompletely resected tumor.

TABLE 1

DOSE SCHEDULE OF PHOSPHORAMIDE DRUGS IN 486 PATIENTS WITH FAR-ADVANCED CANCER

Route	Thio-TEPA	ODEPA	Interval
Intratumor	3–60 mg.	3–80 mg.	1–4 weeks
Intravenous	5–30 mg.	5–40 mg.	1–4 weeks
Intrapleural	10–60 mg.	15–80 mg.	1–4 weeks
Intraperitoneal	10–60 mg.	—	1–4 weeks
Intrapericardial	20–25 mg.	30 mg.	1–4 weeks
Intrahepatic	10–60 mg.	—	1–4 weeks
Intracerebral	10–30 mg.	—	1–4 weeks
Intramuscular	10–20 mg.	—	1–4 weeks
Oral	5–10 mg.	10–20 mg.	1–7 days

Average total dose, 353 mg. (range 20 to 1750 mg. Thio-TEPA and 20 to 3750 mg. ODEPA).

Average duration of therapy, 5.5 months (range 0.2 to 37 months).

Thio-TEPA was used in 380 cases; ODEPA in 26 cases. An additional 80 patients who were being maintained on Thio-TEPA received ODEPA for short periods during shortages of the former phosphoramide. The drug was dissolved in pyrogen-free water, 10 mg. per cc., and sterilized by filtration. It was administered undiluted. The dose schedule employed is indicated in TABLE 1. An attempt was made to treat each patient at the site of disease which posed the greatest threat to his or her well-being or survival. Routes employed included intratumor, intravenous, intrapleural, intraperitoneal, intrapericardial, intrahepatic, intracerebral, intramuscular, and oral. When using the intrahepatic route an effort was made to insert the needle into large metastatic nodules. The intracerebral route was used either for primary or metastatic tumor at the time of surgery or later through a burr hole defect. No side effects were encountered after the latter route except in one instance when a transient convulsive seizure resulted from the injection of a solution too close to the motor cortex. Early in this study it became apparent that larger doses could be used when local injection was employed than when the drug was administered intravenously. It was assumed that a certain portion of the drug was fixed at the injected tumor site. The intravenous route was employed for inaccessible tumor and for maintenance therapy after large local tumors had regressed. Oral medication was used during vacation trips when interruption of therapy was considered undesirable, and in a very limited number of postoperative cases.

The initial dose of drug varied depending on blood count, extent and location of tumor, and the patient's weight. Subsequent doses were guided by the white blood count taken prior to each treatment, as well as by the status of the cancer. The doses of ODEPA were generally 1.5 to 2 times greater than those of Thio-TEPA. When first seen, the patients were treated at weekly intervals and occasionally twice weekly. As disease regressed, the intervals were gradually lengthened to 2, then 3 and, occasionally, 4 or 5 weeks. Patients with lymphomata were seen at longer intervals. Oral tablets were administered more frequently and varied from 1 to 7 times weekly. If therapy was started in the hospital, the patient was ambulated and discharged as soon as possible. Subsequent treatment was administered on an outpatient basis. Patients were re-hospitalized for serious complications or for terminal care, at which time chemotherapy was discontinued.

Results of Therapy

Survival time. Survival time in 486 patients is indicated in TABLE 2. The primary site of disease is indicated in the first column. Patients who died or were lost to follow-up are grouped together. It should be pointed out that all patients including those who expired from their disease within

TABLE 2
SURVIVAL IN MONTHS OF 486 ADVANCED CANCER PATIENTS MAINTAINED ON PHOSPHORAMIDE THERAPY

Tumor	Dead or lost			Alive		
	No.	Avg.	Range	No.	Avg.	Range
Mammary	127	6.9	(0.2–32)	50	10.4	(1–33)
Female genital	59	5.2	(0.5–19)	20	13.1	(1.5–37)
Gastrointestinal	55	3.2	(0.2–18)	4	9.3	(3–17)
Bronchogenic	50	3.4	(0.2–22)	3	4.3	(2–7)
Genitourinary	23	2.6	(0.2–10)	3	4.0	(1–6)
Head and neck	27	2.2	(0.5–14)	1	4.0	(4)
Melanosarcoma	4	3.7	(1–9)	—	—	—
Central nervous system	9	5.8	(0.5–26)	—	—	—
Sarcomas	16	3.4	(0.2–11)	3	9.0	(6–11)
Lymphomata	9	6.2	(1–28)	4	16.3	(6–31)
Miscellaneous	9	4.0	(1–10)	—	—	—
Primary unknown	8	3.4	(0.2–13)	2	2.5	(2–3)
	396	4.7	(0.2–32)	90	10.5	(1–37)

a few days after institution of therapy are included in this group. Data on those patients surviving at this time are given in the last three columns. Since survival time only is hardly worth achieving, some measure of the quality of survival is desirable. Some light may be shed on this point by subtracting the duration of therapy from survival time, since therapy was discontinued when patients could no longer be treated in the clinic and were admitted for terminal care. In the patients with mammary cancer who died, the average duration of survival was 6.9 months; the average duration of therapy was 6.2 months. The difference, namely 0.7 months, may be taken roughly to indicate complete incapacity, and 6.2 months would then represent desirable survival time in the expired group.

The average survival of living patients with mammary cancer is 10.4 months; the range of survival in these patients who are being maintained on phosphoramide therapy is 1 to 33 months. The somewhat better figure for the patients with female genital cancer can be attributed to the good response seen in a large number of cases of ovarian cancer. The longest average survival rate occurred in the lymphomata, a group of neoplastic diseases that frequently respond well to all of the alkylating agents and therefore can hardly be compared to the solid tumors.

Objective results. Objective response repeatedly achieved in a disease in which spontaneous remission rarely occurs must be attributed to therapy. It becomes the more impressive when seen during the florescence of a disease despite energetic conventional treatment.

TABLE 3 summarizes treatment administered to 177 patients with mammary cancer prior to institution of chemotherapy. Mastectomy was performed on 151 patients, a simple procedure being employed in 1 case only; 9 patients had the second breast removed subsequent to the first operation either because of metastatic disease or because a second primary had developed in the opposite breast. Metastatic lesions were treated surgically almost as frequently as by irradiation. Irradiation was used as primary or postoperative therapy in a total of 52 patients. Seventeen were castrated by X rays, 20 by oöphorectomy, and 3 were adrenalectomized. The tumor was out of control following therapy with female hormones in 25 cases and male hormones in 43 cases. A few patients had received a variety of other therapeutic agents including radioactive gold, methyl-bis(β-chloroethyl)-amine (HN2), and triethylene melamine (TEM).

Types of lesions showing varying degrees of regression following chemotherapy with the phosphoramides in patients with metastatic mammary carcinoma are listed in TABLE 4. Ulcerative lesions, primary tumors, and metastatic nodes and nodules were all treated by local injection of the drug and showed an over-all response rate of 66 per cent. Facial edema regressed dramatically when pressure from massive cervical adenopathy was relieved. Serous cavity effusion was treated by instillation of drug through the thora centesis or paracentesis needle. Absence of clinical toxicity rendered this a practical office procedure. Neither "hot" spots as sometimes seen afte Au^{198} instillation nor local tissue reaction such as may follow injection o HN2 occurred. Recalcification occurred in 12 of 32 patients exhibitin

TABLE 3

PREVIOUS THERAPY IN 177 PATIENTS WITH MAMMARY CARCINOMA SUBSEQUENTLY TREATED WITH PHOSPHORAMIDES

Therapy	No. of patients	Therapy	No. of patients
Surgery		Irradiation	119
Biopsy only	26	1 course	52
Mastectomy	151*	2 courses	33
For metastases		3 courses	16
1 time	29	4 courses	11
2 times	11	5 courses	2
3 times	6	6 courses	3
4 times	2	7 courses	1
5 times	1	10 courses	1
6 times	1	Radium	2
Castration		Au^{198}	3
Irradiation	17	P^{32}	1
Oöphorectomy	20	TEM	1
Adrenalectomy	3	HN2	4
Hormones		Cortisone	4
Estrogens	25	Propylthiouracil	2
Androgens	43		

* Nine were bilateral.

TABLE 4

OBJECTIVE RESPONSE TO PHOSPHORAMIDE THERAPY IN 177 PATIENTS WITH ADVANCED MAMMARY CARCINOMA

Total no. patients	Type of lesion	Total improved	
		No.	Per cent
21	Ulceration	14	66
21	Breast tumor	12	57
71	Metastatic nodules	45	64
18	Metastatic nodes	15	80
12	Inflammatory spread	9	75
4	Facial edema	3	75
42	Hydrothorax	33	80
2	Pericardial effusion	2	100
4	Ascites	2	50
32	Osseous metastases	12	37
21	Pulmonary metastases	11	52
12	Central nervous system involvement	8	66
9	Hepatic metastases	3	33

localized osteolytic metastases. However, supplemental irradiation is generally preferred for this complication, since results are more rapid. As in estrogen therapy, nodular pulmonary metastases regressed while those due to lymphangitic dissemination tended to be resistant. Eight of 12 patients with signs and symptoms of central nervous system involvement demonstrated improvement that in some instances was dramatic. Clinically undetectable hepatic metastases are a common finding at post-mortem examination in patients dying with widespread mammary carcinoma. However, a large nodular liver carries a grave prognosis; life expectancy generally is a matter of weeks or, at most, of a few months. No improvement was noted in any patients treated with Thio-TEPA until the drug was injected intrahepatically. The procedure is much the same as needle biopsy of the liver, but is simpler because only a 22- or 23-gauge needle is required.

Case Reports

E. R., a 65-year-old woman, first noticed a lump in the breast in 1945, but not until it ulcerated in 1952 did she seek therapy. A course of X irradiation produced temporary regression. In February 1953, because of recurrent ulceration and the development of a suprasternal mass, the patient was treated with stilbestrol 5 mg. t.i.d. Again there was temporary regression. However, hormone therapy was discontinued in November 1953 because of further extension of disease.

When the patient was first seen, in March 1954, a nodular ulcerated tumor occupied the upper medial quadrant of the left breast and a 4 × 6 cm. thick pancake mass overlay the upper sternum (FIGURE 2a). Biopsy revealed mammary adenocarcinoma. On March 9, 1954, 40 mg. of Thio-TEPA was injected into the breast tumor and this was followed by 10 to 40 mg. given by the same route at 1- to 4-week intervals. By July 14, 1954, after a total dose of 140 mg., the ulceration had healed and there was marked regression of the nodular breast lesion and the suprasternal mass (FIGURE 2b). Therapy was discontinued for 2 months. At this time early progression was noted; therapy was reinstituted and maintained at 3- to 4-week intervals. Again there was regression. In March 1955 (FIGURE 2c) the suprasternal mass was not palpable, and the left breast lesion was replaced by a puckered scar. Subsequently the patient was lost to follow-up.

R. L., a 29-year-old mother of two children, had partial resection of the left breast, followed by X irradiation for metaplastic carcinoma in August 1953. In August 1954 a right radical mastectomy was performed for an acinar cell carcinoma. One year later there was gradual onset of increasing sluggishness, headache, followed by diplopia, intermittent vomiting, and weakness of the right upper extremity. A neurologist made a diagnosis of cerebral metastases. X rays revealed multiple nodular pulmonary densities, and an osteolytic lesion in the right ilium (FIGURE 3a, d). Chemotherapy with Thio-TEPA was instituted in the hospital on February 5, 1956. An initial dose of 30 mg. was given by the intravenous route. This was followed by injections of 5 to 25 mg. given at 1- to 2-week intervals depending on the level of the white blood count. Within 3 weeks after beginning treatment

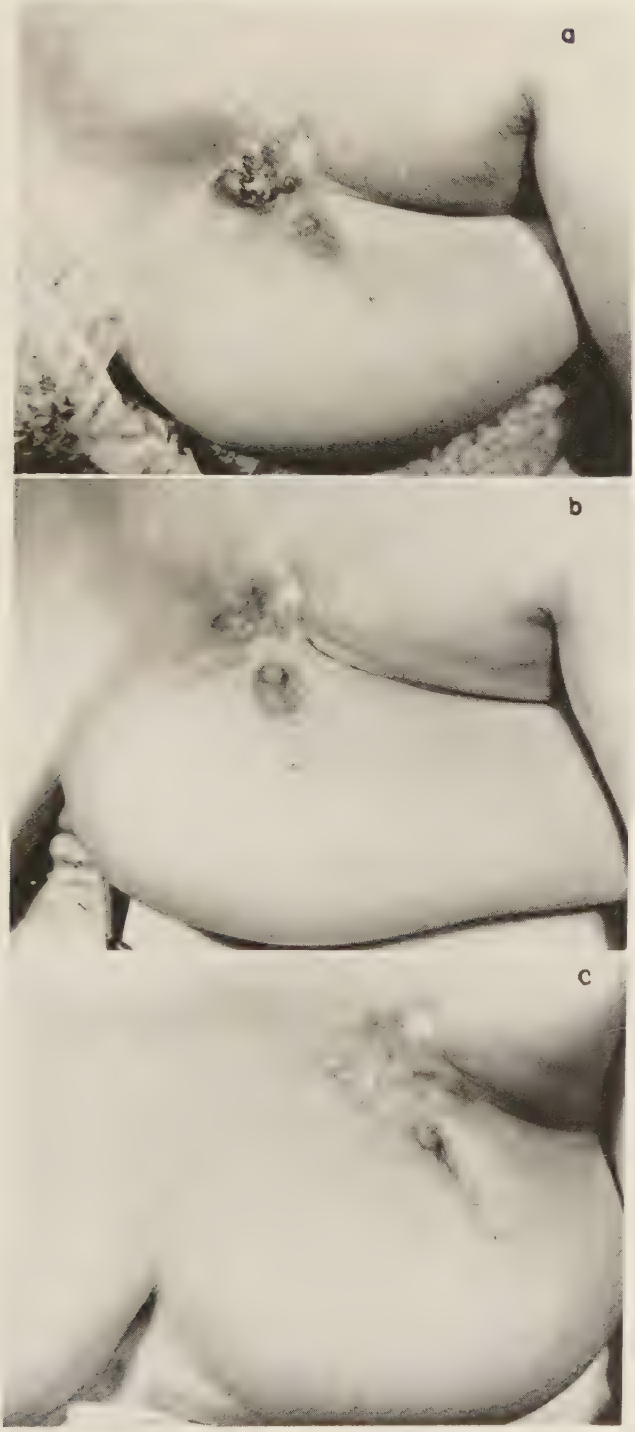

FIGURE 2

the patient was shopping and driving her car. X rays on April 19, 1956, demonstrated regression of pulmonary and osseous lesions (FIGURE 3b, e). On June 21, 1956, after a total dose of 290 mg. Thio-TEPA, the chest X ray showed almost complete disappearance of pulmonary nodules (FIGURE 3c). The patient was permitted to go on a vacation to Atlantic City. After 4½ months of complete clinical remission there was recurrence of cerebral symptoms which did not respond to doses of Thio-TEPA compatible with hematopoietic tolerance. There was a gradual downhill course, and the patien expired on September 30, 1956.

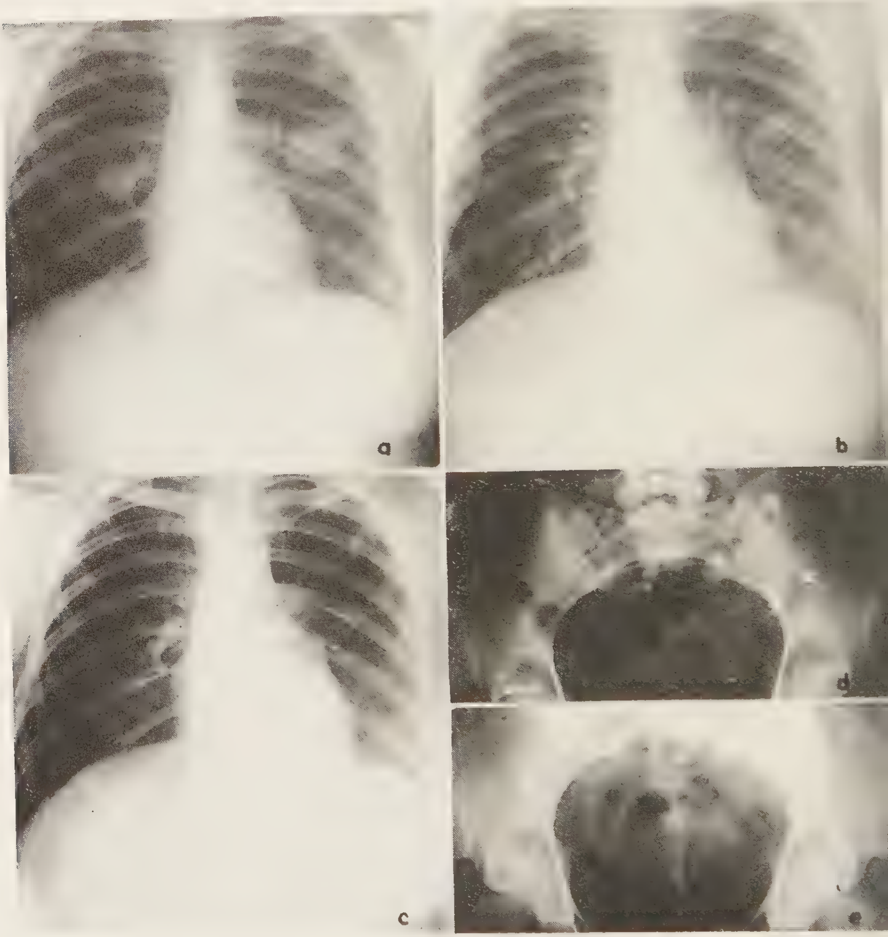

FIGURE 3

TABLE 5 lists demonstrable response to chemotherapy observed in othe types of cancer. As previously reported,[1] Thio-TEPA is an effective palliative agent in a high percentage of cases of ovarian carcinoma. Control o

TABLE 5

OBJECTIVE REMISSION IN FAR-ADVANCED CANCER TREATED WITH PHOSPHORAMIDES

Total pts.	Type tumor	Number of patients improved			
79	Gynecological	Ovarian ca. all types	Epi. ca. cervix	Adenoca. uterus	
	Regression tumor	22	1	3	
	Regression pulmonary nodules	—	—	1	
	Control ascites	6	—	1	
	Control hydrothorax	8	—	1	
	Control pericard. effusion	1	—	—	
53	Bronchogenic				
	Control hydrothorax	1			
	Regression nodes	2			
59	Gastrointestinal	Gastric	Rectal	Pancreatic	
	Regression metastases	1	2	3	
26	Genitourinary	Hypernephroma		Prostatic	
	Regression metastases	—		1	
	Regression pulmonary nodules	1		—	
4	Melanoma				
	Regression metastases	2			
9	C. N. S. tumors				
	Decrease neurological signs	4			
28	Head and neck	Salivary gland	Pharynx	Tongue	Sinus
	Regression tumor	4	4	2	1
9	Miscellaneous	Thyroid	Basal cell		
	Regression metastases	1	—		
	Regression lesion	—	2		
10	Primary unknown				
	Control hydrothorax	1			
	Control C.N.S. signs	1			
19	Sarcomas	Rhabdomyosarcoma	Botryoides sarcoma	Leiomyosarcoma	
	Regression tumor	1	1	2	
13	Lymphoma	Hodgkin's		Lymphosarcoma	
	Regression nodules	3		4	
	Control hydrothorax	—		2	

serous cavity effusion can be achieved easily. Regression of soft-tissue tumor is best accomplished by transabdominal or transvaginal injection of drug. Regression of tumor was achieved in only 1 patient, who had epidermoid carcinoma of the cervix. Slow regression of recurrent or metastatic adenocarcinoma of the uterus was observed in 6 of 11 patients (54 per cent).

Case Reports

M. P., a 69-year-old woman, was first seen in December 1953, following a colostomy for acute intestinal obstruction. In December 1952 a laparotomy had been performed elsewhere for papillary adenocarcinoma of the ovary. Because of vaginal bleeding and a palpable pelvic mass, a second operation was undertaken in April 1954. At that time generalized tumor seeding of the pelvic peritoneum, angulation and infiltration of both ureters, and infiltration of the bladder were noted. Lysis of adhesions and implantation of radon seeds had been performed. In the fall of 1954 symptoms of urinary urgency and frequency as well as signs of progressive bowel obstruction had developed. On December 27, 1953, examination revealed, in addition to the colostomy, a doughy pelvic mass and a small ulcerated oozing area in the vaginal vault. Chemotherapy was instituted with intravenous injections of Thio-TEPA (10 mg. daily for 4 days, followed by 10 mg. weekly). By February 26, 1954, the patient was voiding once every 4 hours instead of once every 1 to 2 hours, and her general condition had improved. Because the pelvic mass now felt hard and nodular, transvaginal injections of Thio-TEPA (20 mg. every 2 weeks) were instituted. By May 1954 the pelvic mass was no longer palpable. The colostomy was closed and normal bowel function with the exception of a tendency to constipation and occasional cramping pains ensued. Since that time and until the present (April 1957) the patient has been maintained on transvaginal injections of Thio-TEPA (5 to 20 mg. given at 3- to 4-week intervals). She has continued to do her own housework and to go on long bus trips. The white blood count has remained between 2000 and 4000. The hemoglobin determination has fluctuated between 8.3 and 12.3 gm. per cent. No transfusions or hematinics have been administered. In the last year the patient has received 20 units of adrenocorticotropic hormone by the intramuscular route once monthly. Antibiotics have been used only occasionally for short episodes of infections unrelated to the tumor. Because discontinuation of chemotherapy has resulted in recurrent disease too frequently uncontrolled in other patients, it has been considered advisable to continue to see and treat this patient at least once a month. Her condition 40 months after institution of maintenance Thio-TEPA therapy is excellent.

P. C., a 35-year-old woman, had a left salpingo-oöphorectomy in Octobe 1953, at which time a dysgerminoma of the ovary was diagnosed. In December 1953 a right salpingo-oöphorectomy and hysterectomy were performed On August 4, 1954, a third laparotomy was undertaken. The pelvis wa found to contain many tumors in the omentum and mesentery, and a larg pelvic mass was fixed to the lateral pelvic walls, the rectum, and bladder Resection of the small bowel was performed because of a 2-cm. tumor implant

The patient was started on transvaginal injections of Thio-TEPA on September 1, 1954. At that time a large firm pelvic mass was palpable. By March 29, 1955, after a total dose of 500 mg. of Thio-TEPA given in doses of 15 to 40 mg. every 1 to 2 weeks, there was no palpable disease and the patient was asymptomatic. Subsequently, she was maintained on chemotherapy given in doses of 5 to 20 mg. at 3- to 5-week intervals. On April 8, 1956 the hemoglobin diminished to 5.9 gm. per cent, the platelets to 14,000, and the white blood count to 3100 per cu. mm. The patient was transfused. Intervals between therapy were increased, and no further hematological difficulty has been encountered. At the present time, 32 months after institution of therapy for rapidly recurring pelvic carcinomatosis, the patient is symptom-free and is working as a laboratory technician.

B. T., a 69-year-old obese woman, developed vaginal spotting and urinary burning in 1951. A dilatation and curettage revealed no evidence of carcinoma. Because of symptomatic uterine prolapse a vaginal hysterectomy was performed. Microscopic section of the surgical specimen revealed adenocarcinoma. In September 1954 there was recurrence of urinary symptoms and pelvic aching. Examination revealed a nodular pelvic mass; a biopsy demonstrated the presence of adenocarcinoma. Contact therapy with radium (1800 mg. hours) was administered. There was partial relief of symptoms. By September 1955 the patient was complaining of severe urinary burning, frequency, and rectal pain. A massive nodular pelvic tumor was palpable. An intravenous pyelogram showed a right hydronephrosis, dilatation of the upper third of the right ureter, and the bladder flattened by an extrinsic mass. The serum nonprotein nitrogen was 52 mg. per cent. On September 14, 1955, the tumor was injected with 40 mg. of Thio-TEPA by the transvaginal route. For the next 6 weeks Thio-TEPA was given in doses of 5 to 25 mg. by the intravenous route. The urinary symptoms improved but, because the large pelvic mass persisted, transvaginal therapy was reinstituted. On December 28, 1955, after a total dose of 395 mg. of Thio-TEPA, the white blood count was 850. Therapy was withheld until January 11, 1956, when the white blood cells numbered 3300 per cu. mm. Thio-TEPA (30 mg.) was injected transvaginally. By January 20, 1956, the white blood count was 1600 and the hemoglobin 6.0 gm. per cent. The patient was transfused and digitalized. Subsequently, chemotherapy was reinstituted and has been maintained at 1- to 5-week intervals in doses of 5 to 15 mg. The severe pain, frequency, and urinary burning disappeared early in the course of treatment. There has been very slow but steady decrease in the size of the pelvic mass. The serum nonprotein nitrogen value returned to normal. By January 30, 1956 the patient had received a total dose of 690 mg. of Thio-TEPA. Occasional transfusions have been necessary. The patient has been on maintenance chemotherapy for 19 months. Except for the first 2 months and an occasional brief period of hospitalization for transfusions, she has been able to carry on mild household activities and appears to be enjoying life with her husband.

Fifty-three patients with bronchogenic carcinoma were maintained on chemotherapy from 1 week to 22 months. Subjective response was occasionally notable, but objective decrease in metastatic nodes was observed in only one patient each with epidermoid and adenocarcinoma of the lung, and diminution in the rate of pleural fluid accumulation was achieved in another whose cell type was not determined.

With the exception of pancreatic tumors and clear-cell carcinoma of the kidney, the objective results of therapy in tumors of the gastrointestinal and genitourinary tract were minimal. Further trials appear to be warranted in the previously mentioned tumors. This is true also in the case of tumors of the salivary glands, thyroid, and central nervous system, wherein unexpected tumor regression or clinical improvement was noted in the limited number of patients who were treated.

Case Report

N. R., an 81-year-old former superintendent of nurses, was admitted to the hospital with hemiplegia and aphasia resulting from a series of cerebral vascular accidents. A mass that had been present on the left jaw for many years proved on biopsy to be mixed-cell tumor of the parotid. The tumo measured 5 × 7.5 cm. in diameter and presented a central draining ulcer A chest X ray revealed multiple metastatic pulmonary nodules (FIGURE 4a) Beginning on June 7, 1955, the tumor on the jaw was injected with Thio TEPA at weekly intervals in doses ranging from 20 to 40 mg. On Augus 16, 1955, the tumor measured 4 × 5 cm., ulceration had healed completely and the chest X ray showed disappearance of two nodules in the right lun and regression of the other nodules (FIGURE 4b). By September 27, 1955 after a total dose of 302 mg. of Thio-TEPA, the mass on the jaw was barel

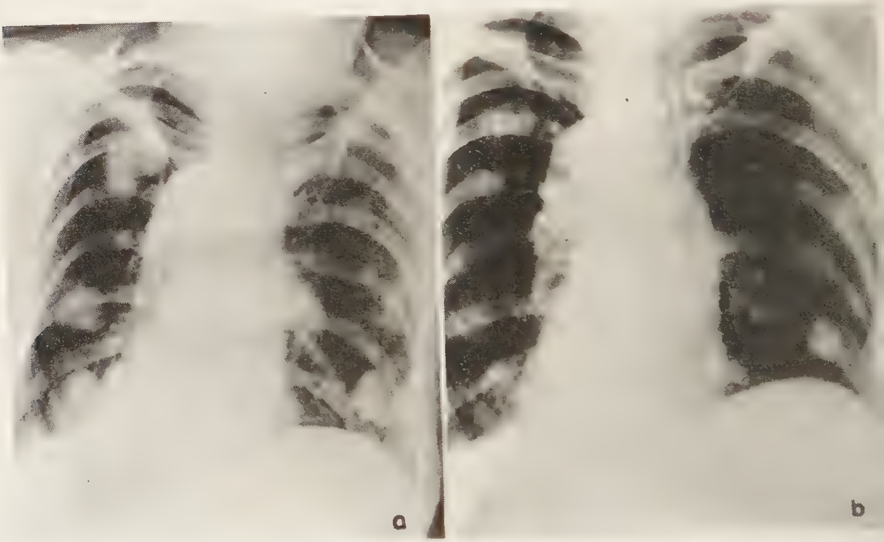

FIGURE 4

palpable. Before another chest X ray could be obtained the patient sustained another cerebrovascular accident and expired.

Thio-TEPA was of definite value in the treatment of the lymphomata. Local masses could be injected when irradiation was contraindicated. It could be used in place of TEM orally, although effects appeared to be somewhat slower than with the melamine. Administration did not require hospitalization.

In a group of 19 patients having a variety of sarcomata, objective improvement was observed in 4 cases only. Many more studies are needed to determine if the natural course of disease in these patients can be altered.

Side Effects

Thio-TEPA and ODEPA differ from most other alkylating agents in that their use is accompanied by minimal or no clinical side effects. The local pain or discomfort noticed on injection of tumor in some cases was probably caused by pressure rather than by either drug per se. This complication could generally be avoided by the use of a small amount of a local anesthetic agent. A few patients noticed a vague sense of depression for 24 to 48 hours following treatment. This was so noticeable in the adrenalectomized patients that their dose of cortisone was increased in the immediate postchemotherapy period.

The phosphoramides, like most of the clinically useful alkylating agents, are hematopoietic depressants. Frequent complete blood counts of patients first treated with Thio-TEPA indicated that leukopenia slightly preceded serious thrombopenia, while the red blood cells were the least and the latest affected. Since accurate white cell counts are much easier and quicker to obtain than platelet counts, this procedure was adopted as a guide to dosage. It was found that when therapy was given no oftener than once weekly, the white cell count was an adequate, safe, and economical guide.

The hematopoietic reserve in patients with widespread cancer varies greatly. It is no doubt affected by previous therapy, especially by X irradiation, as well as by nutritional status. Patients with diminished renal function seem to tolerate less drug than those with good renal reserve. For these reasons therapy must be individualized and constantly adjusted.

The patients in this study had at least a white blood cell count and hemoglobin determination prior to each treatment. In many cases the white blood cell count was kept at a level ranging from 2000 to 3000 for indefinite periods. The leukopenia was not found to be a hazard, especially when the patient could be kept out of the hospital and away from drug-resistant infections, or from exposure to multiple injections not always given with meticulous cleansing at the injection site.

The dosage of drug required to control the disease occasionally resulted in clinical thrombopenia; that is, petechiae, purpura, and epistaxis. As has been pointed out by others, the level of platelet count at which clinical thrombopenia became apparent varied considerably from patient to patient. When these phenomena appeared, administration of the drug was temporarily discontinued, but was resumed as soon as possible. A certain degree of anemia was likewise tolerated. Certainly these manifestations of hematopoietic depression carried nothing like the lethal threat that widespread cancer does. It also should be pointed out that cancer itself can result in anemia as well as thrombopenia and leukopenia when bone marrow becomes involved.

The hematological effects of Thio-TEPA were fairly predictable, but this was not so true with ODEPA. In the case of the latter drug, occasional sudden severe depression of all blood elements took place. Recovery seemed slower than that following depression caused by Thio-TEPA. While chemotherapy was withheld, control of tumor was lost. For this reason Thio-TEPA is considered preferable to ODEPA.

Supplemental Therapy

Blood transfusions were given to these patients only when the hemoglobin determination fell below 7 gm. per cent, except in special cases. Most individuals adjust fairly well to a chronic anemia of moderate severity. This state was considered preferable to the hazards of frequent transfusions. The latter possibility became quite real when early in the study two of our patients expired from homologous serum jaundice following transfusions administered when their hemoglobin levels were above 8 gm. per cent.

Vitamins, with the exception of ascorbic acid, were generally avoided. No particular improvement has been noted in cancer patients who have received large doses of vitamins unless they exhibited specific deficiencies.

Maintenance therapy with small doses of cortisone (12.5 to 25 mg. b.i.d.) and/or adrenocorticotropic hormone (10 to 40 units weekly given by the intramuscular route) was frequently recommended. Earlier studies on patients receiving large doses of HN2 revealed that these hormones did protect the hematopoietic system to some extent. In addition, they improved appetite and well-being in most cases. Only rarely did personality change warrant discontinuation. Electrolytes seemed generally unaffected at this dose level other than for improvement in the frequently subnormal serum sodium level seen in far-advanced cancer patients.

Discussion

Since permanent cure is the exception rather than the rule with cancer, any agent that can control this disease and permit the patient to continue a relatively normal life is a boon. A drug that can be given on an outpatient basis at 1- to 3-week intervals usually permits a patient to continue at work The mere act of getting dressed and making a clinic visit demonstrated t many of our patients that they could be up and about and were not doome to decline slowly and miserably in bed.

The phosphoramides are not curative in far-advanced cancer. For thi reason maintenance therapy becomes imperative. Although eventual dru resistance has been anticipated, no clear-cut case was seen. Patients expire when hematopoietic reserve did not permit continuation of therapy at a leve adequate to suppress the tumor.

The effect on the tumor cannot be achieved unless the drug can reach th tumor in adequate concentration. If a toxic drug is given systemically, much will be dissipated en route to the tumor, and other sensitive systems may be affected. Some tumors are less vascular than neighboring tissues and therefore receive even less drug than does normal tissue. It is probable that speed of fixation in tissue is an important factor as well. Were our present chemotherapeutic agents more specific in action or the tumor cell less like the normal cell, such considerations would be unimportant. Whatever the true explanation may be, it appears clinically that Thio-TEPA and ODEPA attain greater total effectiveness with less hematopoietic damage when given locally.

In summary, the following observations are presented:

(1) Thio-TEPA is most effective in mammary and ovarian carcinoma. Regression was observed in tumors of the pancreas, thyroid, salivary gland, and central nervous system, as well as in adenocarcinoma of the uterus. Slowing of disease and control of pain were achieved in many other tumors.

(2) Thio-TEPA was most effective when given locally, especially into tumor site and intrapleurally. It can be given by any route.

(3) The mass of the tumor is important, since the effect on white blood count and platelets limits the dose.

(4) Drug action is slow. Results of therapy are increasingly apparent over many weeks' time.

(5) Therapy must be individualized for the patient, the type of tumor, localization of the tumor, and extent of the disease.

References

1. Bateman, J. C. & T. Winship. 1956. Palliation of ovarian carcinoma with phosphoramide drugs. Surg. Gynecol. Obstet. **102:** 347–354.

2. Cromer, J. K., J. C. Bateman, G. N. Berry, J. M. Kennelly, C. T. Klopp & L. I. Platt. 1952. Use of intra arterial nitrogen mustard therapy in the treatment of cervical and vaginal cancer. Am. J. Obstet. Gynecol. **63:** 538–548.

OBSERVATIONS ON THE THERAPEUTIC EFFECTIVENESS OF TRIETHYLENE THIOPHOSPHORAMIDE*

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The present study was undertaken to determine whether measurable regression in malignant tumors could be demonstrated following the administration of triethylene thiophosphoramide (Thio-TEPA).

The material contained in this report includes a total of 117 patients with 21 different types of neoplastic diseases. During the course of the investigation the compound was administered intravenously in most of the patients, intramuscularly in a small number, and orally in a few. The dosage was usually initiated with 10 mg. daily for 4 to 7 days and maintained at a rate of 10 mg. once or twice weekly until either (1) a persistent leukopenia below a level of 5000 WBC per cu. mm. developed, or (2) a total of at least 70 mg. of the compound had been administered without detectable objective or subjective response in the patient. The total periods of treatment ranged from 1 to 60 weeks, and the cumulative dosages per patient varied from 30 to 610 mg. Approximately 75 per cent of the patients were able to receive a total dose of 70 mg. or more.

The largest group of patients included 38 with metastatic carcinoma of the breast. Sixteen of these had bone lesions, associated with a severe and disabling pain that had become unresponsive to hormonal management. In 5, or approximately one third of the patients, the pain was rapidly and completely relieved for from 2 to 6 months. At the end of this time, despite continued therapy, the pain recurred, and there was evidence of progression of the bone involvement. In the remaining 11 patients there was either partial or no relief of pain.

In none of these patients did we observe any evidence of recalcification of bone on repeated X-ray examinations. The serum calcium levels were not determined in a consistent manner, since the values obtained prior to the initiation of therapy did not appear to correlate well with the extent of disease observed in the X-ray films.

Nine patients had skin involvement with breast carcinoma, and in only one was an unequivocal objective regression observed.

With regard to other sites of involvement, 3 of 5 patients with pleura effusions showed good responses on maintenance therapy, 2 of 7 patients with hepatic involvement demonstrated definite regression, but in 13 patients with intrapulmonary lymphatic spread no improvement, either objective or subjective, was observed.

Thirteen patients with advanced ovarian carcinoma were treated. In 3 complete, and in 2 almost complete, regression of detectable tumor masse and ascites was observed. One patient who was treated for 1 year an

* The investigation described in this paper was supported by a grant from the Nation Cancer Institute, Public Health Service, Bethesda, Md.

2 months continuously is still free of palpable disease more than 1 year after treatment was discontinued. In 4, recurrence and progression of disease developed while on maintenance therapy.

In 12 patients with lymphomas, only 1, with lymphosarcoma, showed definite regression of disease. In the adenocarcinomas of the intestinal tract, only 1, with metastases from a gastric carcinoma to the liver, demonstrated unmistakable regression of liver size and of jaundice. In pulmonary carcinomas, as well as in scattered instances of melanomas and a variety of sarcomas, no favorable responses were seen.

It has been our observation that there was no correlation between the development of leukopenia and the response to Thio-TEPA therapy. The most favorable results were observed in the patients who did not develop hematopoietic depression and were therefore able to tolerate continued treatment for prolonged periods of time.

In summary, significant measurable regression in certain malignant tumors has been demonstrated in association with the administration of Thio-TEPA. In this series of observations, the highest proportion of such responses occurred in patients with metastatic carcinomas of the ovary and breast. In these two categories of malignant disease, therefore, Thio-TEPA would appear to have significant, although limited, therapeutic effectiveness.

CLINICAL EXPERIENCE WITH TRIETHYLENE THIOPHOSPHORAMIDE, WITH SPECIAL REFERENCE TO CARCINOMA OF THE BREAST*

By George E. Moore

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Eighty-seven patients with advanced metastatic disease (excluding lymphoblastomas) have been treated with at least one course of triethylene thiophosphoramide (Thio-TEPA, TSPA)† comprising five successive daily intravenous injections of 0.2 mg./kg. for a total dosage of 1.0 mg. kg. The majority of these patients were in a randomized series for comparison with actinomycin D. Briefly, there were no clinically significant responses to TSPA among 62 patients with gastrointestinal, lung, or head and neck malignancies. A clinically significant response was defined as a reduction of 50 per cent or more in the size of a majority of measurable lesions for a period of one month. However, temporary measurable tumor regression was seen in several patients, including 2 with lung carcinoma and 1 with an embryonal carcinoma of the testis.

Included in this series were 25 patients with advanced metastatic breast cancer who had become nonresponsive to hormone therapy. These patients had received no specific therapy for about 1 month and had clinical evidence of progressive tumor growth. All had lesions measurable either directly or by X ray. Six patients died before an evaluation period of 1 month was attained (TABLE 1).

TABLE 1
BREAST CARCINOMA TREATED WITH TRIETHYLENE THIOPHOSPHORAMIDE

	Total	Indeterminate	No effect*	Good response
Course I	25	6	10	9
Course II	10	3	1	6
Course III	2	—	1	1

* This group includes patients with remissions of less than 30 days and reduction of lesions less than 50 per cent in diameter.

Of the patients with a good clinical response to TSPA, several deserve special mention since they had a massive visceral involvement that was apparently destroyed by the drug, and because further growth was inhibited for several months. The course of one such patient is outlined in FIGURE 1.

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* The investigation on which this paper is based was supported in part by the Dorothy H. and Lewis Rosenstiel Foundation, New York, N. Y.

† The triethylene thiophosphoramide was obtained from the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y., through the courtesy of J. M. Ruegsegger.

This patient was comatose, in hepatic failure, and had abdominal ascites ecessitating paracentesis every third day. Celiotomy 3 months previously ad shown that at least 70 per cent of her liver was involved with metastases nd that there was massive infiltration of the mesenteries. As is apparent rom FIGURE 1, her improvement after therapy (60 mg. I.V.) was spectacular, nd she was discharged in 3 weeks. This remission lasted 3 months, at which ime multiple skin metastases appeared and grew rapidly.

A second course of therapy (60 mg. I.V.) resulted in complete disappearnce of the metastatic nodules within 2 weeks. X rays revealed definite ealing of pelvic metastases (FIGURES 2 and 3). The patient continued to

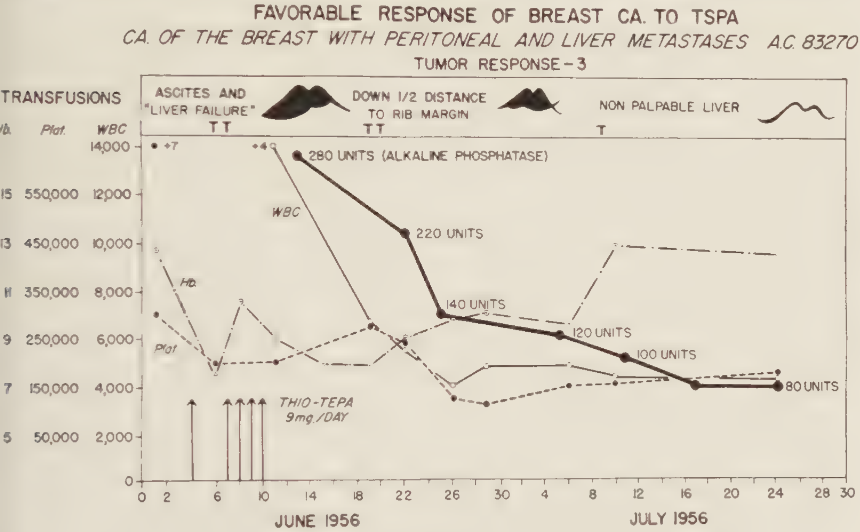

FIGURE 1. Favorable response of breast cancer to triethylene thiophosphoramide.

njoy good health and even participated in competitive bowling. This econd remission lasted 3 months and 1 week, at which time multiple subutaneous metastases again appeared at the same sites previously noted.

A third course of therapy (30 mg. I.V.) resulted in rapid complete disppearance of the nodules of the scalp, face, and neck. However, the atient again had an elevated alkaline phosphatase, formation of ascitic uid, and severe gastric hemorrhages that were thought to be from an ulcerted metastasis of the gastric mucosa.

A fourth course of therapy (30 mg. kg.) was given 2 weeks later, since scitic fluid continued to form and one new subcutaneous metastasis develped over the left mandible. Only a small dose was given because the eripheral white count was 2000. The gastric bleeding ceased, but the rmation of ascites fluid was unaffected. The patient improved and was ischarged from the hospital but within 2 months abdominal masses became alpable, ascites re-formed, and gastric hemorrhage again occurred. At the resent time the patient is hospitalized and seems somewhat improved after rednisone therapy.

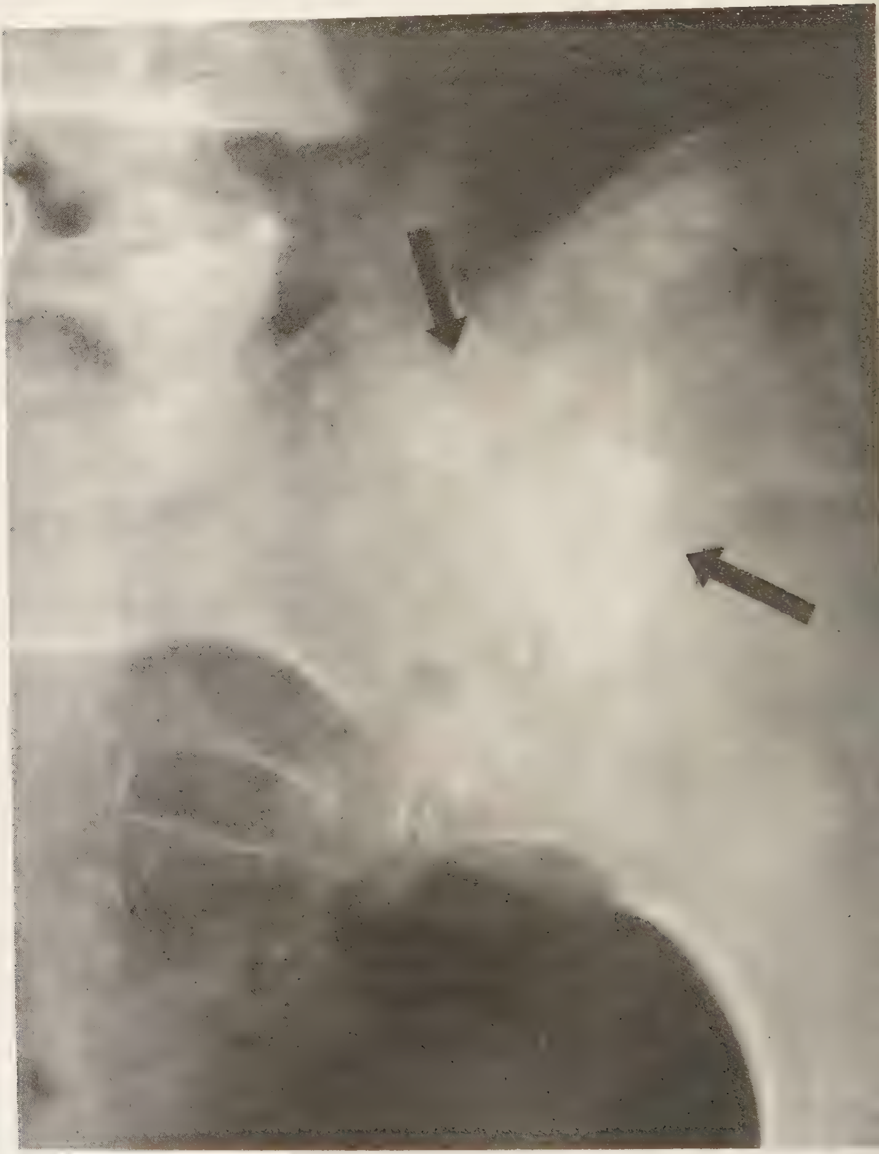

FIGURE 2

Length of Remissions

The clinically significant remissions resulting from a single course of TSPA lasted from 1 to 3 months. Following a second course of therapy abou one half of these patients had a second remission that was identical in length the remainder had shorter periods of remission or did not respond.

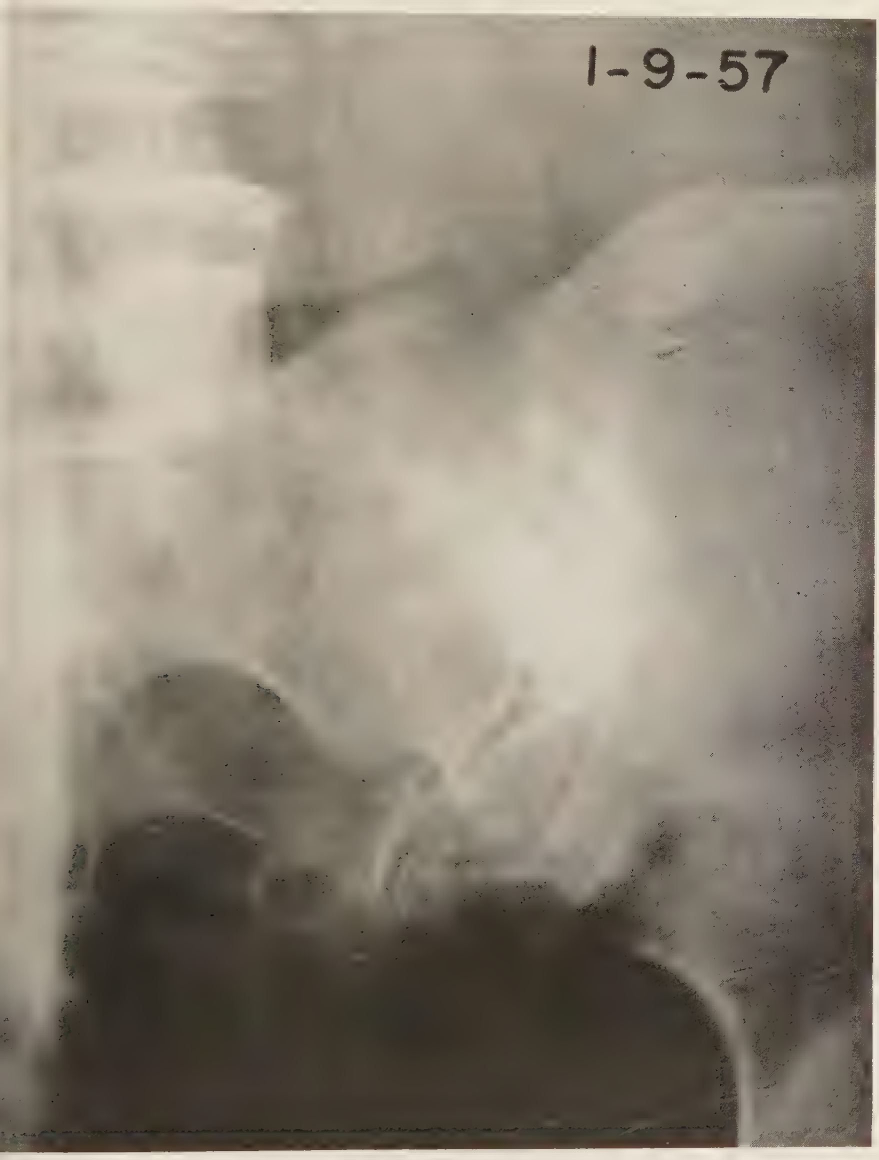

FIGURE 3

Multiple Courses of Therapy

Multiple courses of therapy (intravenous) have been given to patients who failed to respond to the initial course and to those with good clinical response but subsequent recurrent growth. In general, no clinically worthwhile responses were seen in patients refractory to the initial course either at the same or an increased level. In this group, 2 of 10 patients died from complications attendant to depression of the blood elements. All patients who responded initially also responded to a second course of 1 mg. kg. given intravenously, but effective clinical response was obtained in only one half of the patients. Because of bone marrow depression, few patients were able to undergo a third course of therapy.

Maintenance Therapy

Following an initial course of TSPA, a few patients were "maintained" on a single intravenous injection of 0.2 mg. kg. given weekly. It was our impression that this method was not as effective in prolonging remissions as spaced courses of therapy and that, in fact, it made it difficult to give subsequent effective amounts of TSPA because of bone marrow depression.

Sites of Metastases

The responses of various metastases according to site are shown in TABLE 2.

TABLE 2
METASTATIC SITES AND RESPONSE TO TSPA

Site of lesion	No response	Response	Duration of response (months)
Skeletal	9	1	9
Soft tissue	10	9	2–9
Pleural effusion	3	3	2–6–4
Abdominal ascites	1	1	7

It must be emphasized that these remissions are the result of systemic therapy only. Too few patients were given local and intracavity therapy to be worthy of comment.

Chemical Changes

All routine chemistries were performed without observing striking changes even accompanying the remissions in the breast cancer series. The only exceptions were alkaline phosphatase and serum bilirubin determinations in patients with liver involvement (FIGURE 1).

Since several groups have suggested that the good effects of TSPA may be limited to lesions responsive to adrenal depression, the adrenal capacity test described by Sandberg[1] was given to 9 patients before and after TSPA. No evidence of depressed function of the adrenal was found.

Toxicity

Almost all serious and fatal complications of TSPA therapy resulted from hematopoietic depression. TABLE 3 indicates the extent of the leukopenia and thrombocytopenia following a single intravenous course of 1.0 mg. kg. of TSPA in the breast carcinoma series. Of the entire group of 62 patients with other solid tumors, 18 had a depression of their peripheral white cell

TABLE 3
TOXICITY TO INITIAL COURSE OF TSPA (1 MG./KG./I.V.)

Patients	WBC below 3000	WBC below 1500	Plat. below 100,000	Plat. below 50,000
Carcinoma of breast 25	9	6	10	3
Other solid tumors 62	18	5	29	11

count below 3000, and 5 of these were below 1500. In 29 patients, the platelet count was depressed below 100,000, and in 11 below 50,000.

Maximal depression of peripheral blood elements occurred 2 weeks following the last injection day: leukocytes, range 5 to 30 days, median 15 days; platelets, range 4 to 28 days, median 17 days.

Patients with extensive liver metastases were most susceptible to depression of the peripheral cellular elements.

Infection accompanied leukopenia and was the single most important cause of death. Two patients with metastatic carcinoma involving the large bowel and liver died of gas gangrene. Other patients also were unable to control the growth of saprophytic organisms.

Despite low platelet counts (as low as 12,000 after second course), bleeding was not encountered in the breast cancer series. In the patients with solid tumors, however, the presence of exfoliating and ulcerating malignant tissue plus therapy did result in fatal hemorrhage.

TABLE 4
DEATHS WITHIN 45 DAYS AFTER TSPA THERAPY IN 25 PATIENTS WITH BREAST CARCINOMA

	First course (1 mg./kg.)	Second course (1.5 mg./kg.)	Third course (1.0 mg./kg.)
Total patients/deaths	25/10	10/6	2/1
Not a factor	8	4	0
Indirect factor	1	0	0
Direct factor	1	2*	1

* One patient had depressed marrow from maintenance therapy before second course.

One patient with metastatic breast carcinoma involving the trachea had a regression of her lesions, but concomitantly developed a tracheal-esophageal fistula followed by aspiration pneumonia and death. In the solid-tumor groups several similar episodes occurred.

Fatalities occurring within 45 days subsequent to TSPA were uncommon except in patients with extensive liver involvement or in patients receiving a

TABLE 5

DEATHS WITHIN 45 DAYS AFTER TSPA IN 62 PATIENTS WITH SOLID TUMORS

	First course (1.0 mg./kg.)	Second course (1.0 mg./kg.)
Total patients/deaths	62/45	3/2
Not a factor	39	1
Indirect factor	1	1
Direct factor	6*	1

* Three patients had over 80 per cent involvement with metastases.

second or third course of therapy. TABLE 4 depicts the fatalities in the breast cancer series and TABLE 5 the other solid-tumor patients.

Pathological Changes

Most patients had serial biopsies, which revealed various degrees of cellular changes, such as swelling, vacuolization, pyknotic nuclei, and abnormal giant cells. In some patients with good clinical response to TSPA, few pathological changes accompanied the gross disappearance of tumor nodules.

Peripheral Blood Studies

Interestingly enough, study of serial cellular concentrates from the peripheral blood showed a marked decrease in the number of tumor cells within 48 hours. In contrast to an average slide having 50 to 200 tumor cells before treatment, the slides of several patients obtained 48 hours after therapy had only 10 to 20 cells, and those from the 4th to the 14th day, only one or no cells per slide. Whether or not such observations will be of importance in evaluating chemotherapeutic effects is not known at present.

Study of these special slides from some patients also revealed an intense vacuolization of the neutrophils after TSPA therapy that often lasted for several weeks. If this effect is substantiated by additional studies it may represent a toxic reaction such as has been described after irradiation. No comparable changes were noted in the lymphocytes and the tumor cells.

Acknowledgment

I express my appreciation to William H. Wehr, Kumao Sako, and to Robert C. Boggess for their assistance.

Reference

1. SANDBERG, A. A., K. EIK-NES, L. T. SAMUELS & F. H. TYLER. 1954. Effects of surgery on blood levels and metabolism of 17-hydroxycorticosteroids in man. J. Clin. Invest. **33:** 1509–1516.

CLINICAL STUDIES OF TRIETHYLENE THIOPHOSPHORAMIDE IN THE TREATMENT OF ADVANCED NEOPLASTIC DISEASE*

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The ethylenimine derivative triethylene melamine (2,4,6-triethylenimino-*s*-triazine, TEM) has received thorough clinical study of its nitrogen-mustard-like effect on neoplastic disease in animals[1-4] and man.[5-8] Its relatively insignificant immediate side effects and versatility of administration, as well as therapeutic results entirely comparable with those of nitrogen mustard, stimulated interest in chemically related agents. The possibility that such agents might exceed the therapeutic range of nitrogen mustard was suggested when certain epithelial neoplasms appeared to respond transiently to TEM.[9] Subsequently the phosphoramides diethylene phosphoramide (DEPA) and triethylene phosphoramide (TEPA) were given clinical trial.[10,11] Although it has been suggested that the treatment spectrum of TEPA is broader than that of nitrogen mustard or TEM,[11] this has not been clearly demonstrated.[10] Clinical studies of the phosphoramide N,N',N''-triethylene thiophosphoramide (Thio-TEPA) have indicated an effectiveness against chronic granulocytic leukemia and chronic lymphocytic leukemia comparable to that of TEM.[12-14] Additional trials of Thio-TEPA suggest that this agent may have definite, although limited, usefulness in the control of advanced carcinomatous disease.[15-17] The ability to administer this agent effectively by the oral, intravenous, intramuscular, intracavitary, intra-arterial, and interstitial routes has been demonstrated.[16-18]

The usefulness of the alkylating agents in the treatment of advanced neoplastic disease is extremely limited. That these chemicals derive their therapeutic value from their partially understood cytotoxic activity results in considerable risk in administration in high dosage. This is especially true in the treatment of advanced carcinomatous disease in which the tumor consists of a highly resistant cell population whose predecessors had been only slightly sensitive to early modalities of treatment (for example, irradiation, hormonal treatment, or surgery). The sometimes striking effectiveness of nitrogen mustard-like agents in lymphoma-leukemia disease is most likely the result of the close relationship of these conditions to the reticuloendothelial system, which is especially sensitive to these chemicals. Therefore, a partial regression of such neoplasms can be expected in some instances after very modest dosage. This cannot be expected to occur in epithelial tumors, especially if cellular differentiation is present to any degree. The need is clear for administration of the maximum tolerated dose in every case in as short a time interval as possible.

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The present study has two chief objectives: first, to determine the largest dose of Thio-TEPA that will elicit a white blood cell (WBC) and/or platelet decline below 5000/cu. mm. and 100,000/cu. mm., respectively, in the shortest and safest time interval; second, to determine the therapeutic effectiveness in relationship both to the "loading" dosage and to the total dose given.

Materials and Method

Thirty-nine adult patients with advanced cancer or lymphoma disease (including one patient with chronic lymphocytic leukemia) were treated with Thio-TEPA through the intravenous or intracavitary route. TABLE 1 indicates the distribution of neoplasms studied and the routes of administration of Thio-TEPA. Alkylating agents had been given previously to 4 and radiotherapy to 9 of the lymphoma patients. Although none of the 28 nonlymphomatous tumors had been treated with nitrogen-mustardlike substances, 21 had been irradiated. Patients were admitted to the study after observation for not less than 2 weeks indicated objective progression of disease. No other form of therapy had been received for a period of at least 2 months and all patients possessed a WBC above 5000 and a platelet count above 100,000. Bone marrow studies were carried out only when peripheral hemoglobin, WBC, or platelet count indicated possible bone marrow depression. The patients were observed at semiweekly intervals during the first 2 weeks and, later, at weekly intervals, when measurable masses were carefully recorded on a standard diagram. The white blood count and platelet count were recorded at each visit, and hemoglobin and differential white blood

TABLE 1
THIO-TEPA: TYPES OF NEOPLASM AND ROUTES OF ADMINISTRATION

	Intravenous	Intrapleural	Intrapericardial	Intraperitoneal	Number
Carcinoma breast	5	3			8
Bronchogenic carcinoma	6	1	1		8
Hodgkin's disease	2	1			3
Lymphosarcoma	3				3
Reticulum cell sarcoma	4				4
Chronic lymphocytic leukemia	1				1
Carcinoma rectum	1	1			2
Carcinoma cervix	2				2
Carcinoma ovary	1	1			2
Teratoma ovary				1	1
Carcinoma stomach				1	1
Carcinoma tongue	1				1
Carcinoma kidney	1				1
Fibrosarcoma	1				1
Carcinoma pancreas		1			1
Total	28	8	1	2	39

counts were carried out weekly or semiweekly as required. When indicated, X-ray examinations were made to observe changes in mass size not accessible to palpation. Patient activity was recorded in terms of "performance status" as described by Karnofsky.[6]

Subjective improvement (S.I.) was considered to have occurred when the patient unequivocally stated an improved feeling of well-being. This was measured by disappearance of such symptoms as nausea, anorexia, easy fatigability, weakness, sweating, pruritis, and pain. Although definite S.I. may have been recorded, this does not imply increased performance status (P.S.), for in many instances improvement in a fraction of the symptomatology was not accompanied by increased physical activity. Therefore, S.I. is recorded as Poor (P), Fair (F), Good (G), Excellent (Ex.) in FIGURES 1 and 2; attempts were made to quantitate as nearly as possible the proportion of relief of symptoms as described above. Objective improvement is expressed in like manner, with a score of Excellent (Ex.) indicating complete disappearance of measured objective findings and without the simultaneous appearance of new masses. A score of Fair (F) implies approximately 50 per cent reduction of mass size.

Crystalline Thio-TEPA* in bulk was stored under refrigeration and dissolved as needed in the amount of 2 mg. per 1 cc. of sterile saline for immediate injection. The intravenous schedule employed, in all but 5 patients who were treated during the early phase of the study, was 20 mg. injected at 3- to 4-day intervals for 3 to 5 doses. This is the maximum tolerated single dose and was given regardless of weight. It represents a dosage of from 0.66 mg. to 1.80 mg./kg. of body weight in the initial 2-week (loading) dosage in this series. Maintenance treatment, defined as the dosage required to elicit intermittent hematological depression over an indefinite period, was given to all patients in 10, 20, or 30 mg. doses at semiweekly, weekly, or semimonthly intervals.

The intracavitary treatment schedule ultimately adopted was 50 mg. of Thio-TEPA in 25 cc. normal saline injected immediately following aspiration of effusion. This dose, repeated in 1 week and at the end of the second week f necessary, represents a dosage of from 1.54 to 2.50 mg./kg. of body weight n a 1- to 2-week period. Treatment was discontinued when fluid formation eased or when the depression of WBC or platelets contraindicated further herapy.

The ranges of total dose and duration of treatment (TABLE 2) were both ery wide. The wide effective total-dose range in the intravenous and intrapleural groups reflects to some degree the variation in hematological tolernce. The somewhat higher total dose in intravenous therapy reflects naintenance over a long period of time as opposed to shorter courses of reatment in the intracavitary group. Although, in general, greater ematological tolerance may be expected from intracavitary treatment, as uggested in TABLE 2 by the dose-time ratio in that group, nevertheless, ndividual patient tolerance cannot be predicted, and occasionally relatively

* Thio-TEPA was generously supplied by J. M. Ruegsegger, Medical Research Section, ederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

TABLE 2
THIO-TEPA: RANGE OF TOTAL DOSE AND DURATION OF THERAPY

Route of therapy	Number	Range total dose (mg./kg.)	Range duration of treatment (months)
Intravenous	28	0.4–5.3	0.25–8.0
Intrapleural	8	1.2–4.4	0.25–3.75
Intraperitoneal	2	0.9–1.5	0.25–1.0
Intrapericardial	1	1.4	1.5
Total	39		

small intracavitary doses (1 mg./kg. or below) have resulted in WBC and/or platelet depression.

Results

Twelve of the 28 patients receiving intravenous Thio-TEPA experienced subjective improvement. Six of these demonstrated definite measurable regression of mass size (FIGURE 1) and are considered to be examples of "objective improvement." However, in terms of "performance status," only one patient improved strikingly, demonstrating an increase from 30 per cent to 90 per cent. Since this chart was made, one patient with carcinoma of the ovary has attained an improvement from 60 per cent to 90 per cent. It is noted further in FIGURE 1 that 5 of the 6 objective therapeutic responses lie evenly distributed in the 2-week dose range of 0.9 to 1.8 mg./kg. of body weight. Those patients considered to have only subjective improvement lie chiefly below the 0.9 mg./kg. level and overlap to some degree into the objective-improvement group.

Of the 28 patients receiving intravenous therapy, 17 (60 per cent) experienced a significant drop in the WBC and/or the platelet count in the initial 2-week dosage period, as shown in FIGURE 1. The 11 2-week blood-response failures are evenly distributed throughout the dose range. However, it is of interest that approximately 67 per cent of the patients in the more successful therapeutic range, above 0.9 mg./kg., experienced a blood drop, while 50 per cent responded in the lower range. Of the 12 patients gaining subjective or objective improvement, only 2 failed to obtain a blood response after the initial loading period and before the onset of maintenance therapy

A significant combined WBC and platelet drop occurred in 10 patients a WBC drop alone in 6; and a platelet depression alone in 1 after the 2-week dose. In all instances except 2, the WBC dropped below the critical leve prior to the platelets, and in a time range of from 4 to 9 days after the completion of the initial dosage period in a majority of patients. The time interval for platelet depression was less uniform and ranged from 7 to 20 days

In FIGURE 2 the total Thio-TEPA dose is compared with the ultimate blood response and with the therapeutic result. Twenty-four of 28 patients demon strated adequate WBC and/or platelet depression, and only 1 showed platelet effect alone. The blood-response failures lie entirely below the dose level dividing the objective-improvement group from those patients experiencing only subjective improvement. All patients demonstrating S.I. or O.I. attained a satisfactory depression of the WBC and/or platelet count.

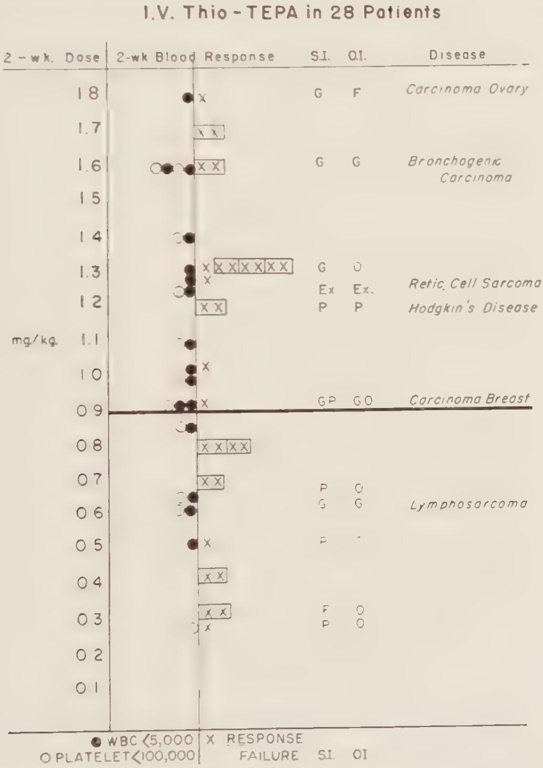

FIGURE 1. Relates the 2-week dose and 2-week blood response to the degree of subjective improvement (S.I.) and objective improvement (O.I.). Instances of objective improvement are identified by diagnosis. Failure of WBC and platelet response is indicated by XX. X indicates failure of WBC or platelet response to drop below 5000 or 100,000, respectively.

Further study of FIGURE 2 suggests that the therapeutic response depends on the total dose given, inasmuch as the 6 patients attaining only subjective improvement received less than 2 mg./kg. of body weight. However, the duration of treatment indicates whether there was initial improvement, which then continued during the greater part of the maintenance therapy. This also indicates the degree of the patient's hematological tolerance to the agent over an extended period of time. The patients attaining only subjective improvement and the majority of complete therapeutic failures tolerated only lower loading doses, primarily because of earlier and more pronounced hematological depression. Therefore, the failure of the group below the 2.0 mg./kg. level to attain a better improvement rate is more closely related to factors controlling the tolerated level of loading (2-week) dose than to the total dose given. It is most likely that prolonged therapy, when it can be tolerated, aids in completing or maintaining improvement initiated by the loading dose. The duration of remission is reflected roughly by the duration of treatment, which was discontinued when the disease again began to progress (FIGURE 2). It is suggested that a maximum safe initial intravenous dosage should lie between 0.9 and 1.8 mg./kg. of body weight when the material is given over a 10-day to 2-week period. Maintenance therapy must be given in sufficient quantity to continue an intermittent depression of white and platelet blood elements. Finally, it is suggested that a maximum tolerated individual intravenous dose must be used rather than a dose based on weight alone and derived from the average dose tolerated in some previous experience.

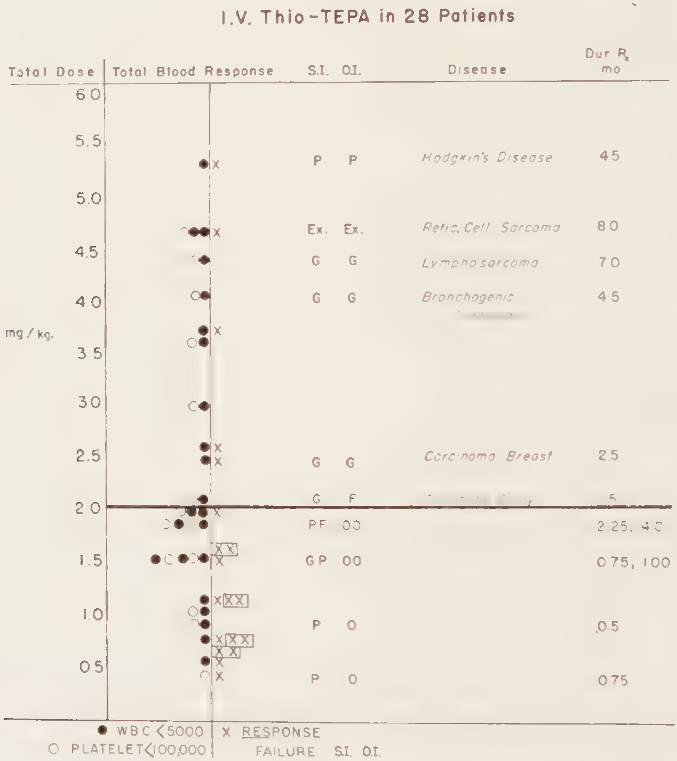

FIGURE 2. Relationship of total dose and total (ultimate) blood response to degree of S.I. and O.I.

FIGURE 3 represents the blood response in a 51-year-old white male with bronchogenic carcinoma of 15 months' duration. Two courses of radiotherapy had been given the last terminating 2 months prior to Thio-TEPA treatment without beneficial effect. Mediastinal obstruction with edema of head and neck tissues, venous engorgement, and hoarseness were marked. Thio-TEPA was given intravenously in doses of 20 mg. each at 3- to 4-day intervals for 10 days, representing an initial dosage of 1.6 mg. kg. of body weight. A significant drop in WBC occurred 10 days after the initial dosing was completed, and in the platelet count in 17 days. Improvement in symptoms and in measurable objective signs occurred in 1 to 2 weeks, and stability in blood elements reappeared in 3 weeks, when maintenance treatment was begun. Maintenance treatment once again resulted in a drop of WBC and platelet count below critical levels. After 4.5 months of therapy all signs and symptoms recurred, although the WBC and platelet count remained below 5000 and 100,000 respectively, and Thio-TEPA was discontinued.

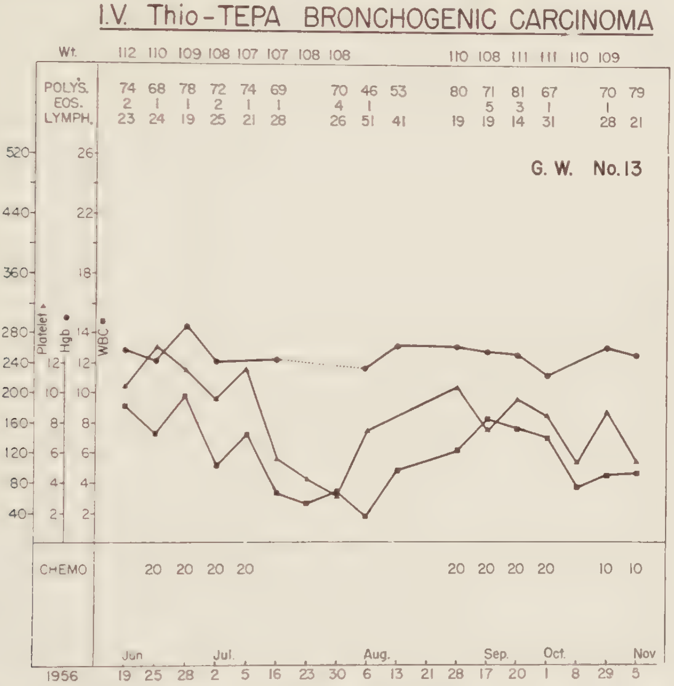

FIGURE 3. Pattern of WBC and platelet response to 10 day course of Thio TEPA therapy, and maintenance therapy with Thio-TEPA.

An occasional patient demonstrated remarkable resistance to Thio-TEPA in both hematological and therapeutic response. J. W. (FIGURE 4) is a 41-year-old white male with Hodgkin's disease of 4 years' duration. He had received radiotherapy with satisfactory response on several occasions, the last course of treatment having been terminated 22 months before Thio-TEPA therapy. He had diffuse adenopathy, splenomegaly, anorexia, fever and night sweats, weakness, and polymorphonuclear leukocytosis. He received 100 mg. Thio-TEPA intravenously in 20-mg. doses at 3- to 4-day intervals for 13 days, representing a total dose of 1.2 mg./kg. of body weight. Because of failure of WBC or platelet response the dosage was continued as indicated on the graph, without definitive blood depression. On two occasions a 30-mg. dose was injected. It is noted that no change occurred in the differential WBC during the period charted. Although fever and night sweats disappeared, no significant objective remission occurred. Nodes and spleen increased or decreased in size in sporadic fashion so that no definite trend could be recorded. This patient was considered to have attained a subjective improvement of limited degree, and at the present time he continues to maintain a performance status of about 70 per cent, which is unchanged since the onset of treatment, now in its sixth month.

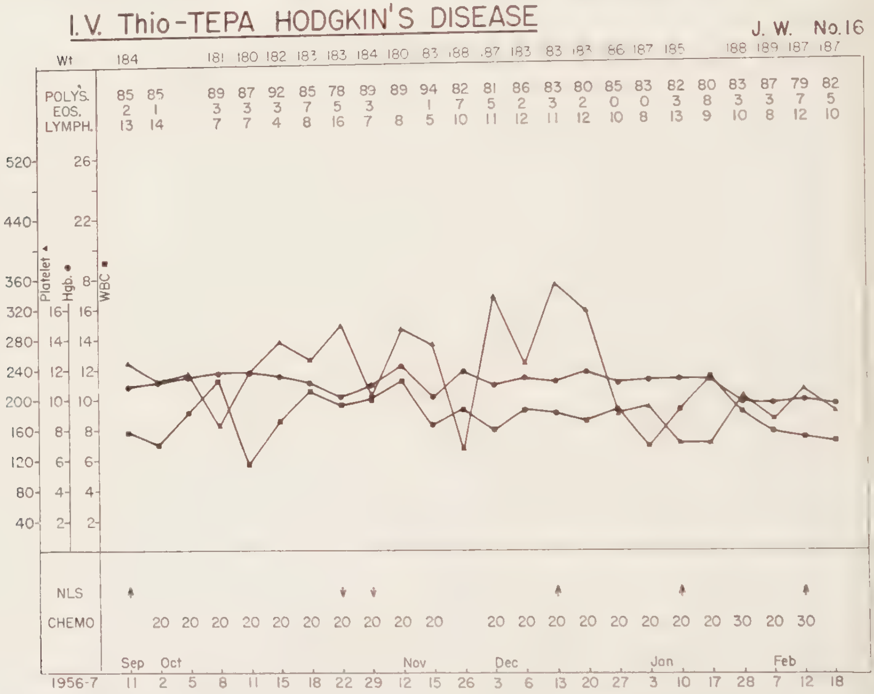

FIGURE 4. The hematological resistance of one patient to Thio TEPA is demonstrated. NLS = nodes, liver, spleen.

Intracavitary Thio-TEPA was administered to 11 patients. Eight of these received intrapleural treatment, and are represented on TABLE 3. Two patients failed to attain a satisfactory blood depression and died before adequate treatment could be given. It is of interest that in this small series a blood response was observed as frequently in intracavitary treatment as in intravenous administration. In one patient with advanced breast carcinoma involving the skin of the chest and with pleural effusion, a satisfactory reversal of the skin lesions occurred following intrapleural therapy. One patient with bronchogenic carcinoma and pericardial effusion with tamponade was treated with 60 mg. of Thio-TEPA in the pericardial space over a 2-week period, with complete resolution of the effusion. This patient died within 2 months after treatment from the encroachment of the primary complex, but no further pericardial effusion was noted. Two patients with diffuse abdominal carcinomatosis from ovarian carcinoma and gastric carcinoma failed to respond after intraperitoneal therapy.

TABLE 3
INTRAPLEURAL THIO-TEPA IN 8 PATIENTS

Dosage		Blood		Duration of therapy	Improvement		Disease
mg./kg.	Total mg.	WBC	Plat.	wk.	S.I.	O.I.	
1.22	90	×	×	3	0	0	Ca. pancreas
1.30	90	●	×	4	F	F	Ca. ovary
1.54	100	●	○	2	P	P	Hodgkin's dis.
1.80	100	×	×	2	0	0	Ca. rectum
2.50	150	●	○	1	Ex.	Ex.	Ca. bronchus
3.00	150	●	○	2	G	G	Ca. breast
3.70	315	●	○	15	Ex.	Ex.	Ca. breast
4.40	220	●	×	6	F	F	Ca. breast

Symbols: × = failure of WBC depression below 5000, or platelet depression below 100,000; ● = WBC below 5000; ○ = platelets below 100,000.

Toxic Effects

No immediate toxic side effects were noted except for occasional anorexia in three patients and slight nausea in one. One patient complained of a "metallic taste" in the mouth after each injection. No evidence of severe bone marrow depression with purpura was noted in this series. Prolonged depression of the platelet count occurred, however, and it is also to be noted that in approximately 50 per cent of instances the platelet count accompanied the white count depression in all modes of therapy.

Conclusions

(1) Intravenous Thio-TEPA is of limited value in the subjective palliation of advanced carcinomatous disease, particularly of bronchus and breast. Excellent objective improvement is uncommon and is usually of short duration.

(2) Thio-TEPA does not appear to exceed nitrogen mustard in its effect on lymphomatous disease. A study of comparative effect should be carried out in order to establish the relative value of Thio-TEPA and other alkylating agents in this respect.

(3) Although prediction of therapeutic outcome cannot be made, it appears from this study that the therapeutic response is dependent more on the toler-

ated initial dosage level than on the total dose given. This is especially important in treatment of epithelial tumors where hematological and tumor cells demonstrate wide variance in their relative sensitivity to the cytotoxic effects of the agent.

(4) Thio-TEPA is of definite value in the palliative control of intracavitary effusions secondary to diffuse carcinomatous or lymphomatous disease.

References

1. Burchenal, J. H., M. L. Crossley, C. C. Stock & C. P. Rhoads. 1950. The action of certain ethylenimine (aziridine) derivatives on mouse leukemia. Arch. Biochem. **26**: 321–323.
2. Burchenal, J. H., S. F. Johnston, M. A. Cremer, L. F. Webber & C. C. Stock. 1950. Chemotherapy of leukemia. V. Effects of 2,4,6-triethylenimino-s-triazine and related compounds on transplanted mouse leukemia. Proc. Soc. Exptl. Biol. Med. **74**: 708–712.
3. Sugiura, K. & C. C. Stock. 1952. Studies in a tumor spectrum. II. The effect of 2,4,6-triethylenimino-s-triazine on the growth of a variety of mouse and rat tumors. Cancer. **5**: 979–991.
4. Sparks, B. S., M. L. Stevens, J. M. Landes, S. L. Halliday, D. McKenzie & J. H. Williams. 1953. The effect of a series of ethylenimine derivatives on myeloid chloroleukemia in the rat. Blood. **8**: 655–660.
5. Rhoads, C. P., D. A. Karnofsky, J. H. Burchenal & L. F. Craver. 1950. Triethylene melamine in the treatment of Hodgkin's Disease and allied neoplasms. Trans. Assoc. Am. Physicians. **63**: 136–146.
6. Karnofsky, D. A., J. H. Burchenal, G. C. Armistead, Jr., C. M. Southam, J. L. Bernstein, L. F. Craver & C. P. Rhoads. 1951. Triethylene melamine in the treatment of neoplastic disease. A.M.A. Arch. Internal Med. **48**: 477–516.
7. Rundles, R. W. & W. B. Barton. 1952. Triethylene melamine in the treatment of neoplastic disease. Blood. **7**: 483-507.
8. Kravitz, S. C., H. D. Diamond & L. F. Craver. 1952. The clinical use of triethylene melamine. Blood. **7**: 729–742.
9. Sykes, M. P., R. W. Rundles, V. K. Pierce & D. A. Karnofsky. 1955. Triethylene melamine in the management of far advanced ovarian cancer. Surg. Gynecol. Obstet. **101**: 133–140.
10. Sykes, M. P., D. A. Karnofsky, F. S. Philips & J. H. Burchenal. 1953. Clinical studies on triethylenephosphoramide and diethylenephosphoramide, compounds with nitrogen mustard-like activity. Cancer. **6**: 142–148.
11. Farber, S., R. Appleton, V. Downing, F. Heald, J. King & R. Toch. 1953. Clinical studies on the carcinolytic action of triethylenephosphoramide. Cancer. **6**: 135–141.
12. Shay, H., C. J. D. Zarafonetis, N. Smith, I. Waldow & D. Sun. 1953. Treatment of leukemia with triethylenethiophosphoramide (Thio-TEPA). A.M.A. Arch. Internal Med. **92**: 628–645.
13. Zarafonetis, C. J. D., H. Shay & D. C. H. Sun. 1955. Triethylenethiophosphoramide in the treatment of chronic leukemia. Cancer. **8**: 512–522.
14. Silverberg, J. H. & W. Dameshek. 1952. Use of triethylenemelamine in treatment of leukemia and leukosarcoma. J. Am. Med. Assoc. **148**: 1015–1021.
15. Shay, H. & D. C. H. Sun. 1955. Clinical studies of triethylenethiophosphoramide in the treatment of inoperable cancer. Cancer. **8**: 498–511.
16. Bateman, J. C. 1955. Chemotherapy of solid tumors with triethylenethiophosphoramide. New Engl. J. Med. **252**: 879–887.
17. Bateman, J. C. & H. N. Carlton. 1956. Palliation of mammary carcinoma with phosphoramide drugs. J. Am. Med. Assoc. **162**: 701–702.
18. Bateman, J. C., B. Moulton & N. J. Larsen. 1955. Control of neoplastic effusion by phosphoramide chemotherapy. A.M.A. Arch. Internal Med. **95**: 713–719.

DIRECT INSTILLATION OF NITROGEN MUSTARD IN THE MANAGEMENT OF MALIGNANT EFFUSIONS*

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Effusions into the pleural, pericardial, or peritoneal cavities as the result of metastatic carcinoma may present difficult problems in management. Until comparatively recently, X-ray therapy was the only method available for the treatment of these malignant effusions. Although such therapy may be effective it is often poorly tolerated. The treatments usually require several weeks and are frequently complicated by radiation sickness. It is difficult to treat the entire area of the pleura or peritoneum, and damage to the skin and contiguous tissues limits the amount of radiation that can be employed.

Following the introduction of radioactive isotopes for intracavitary use,[1, 2] this method gained ready acceptance in the management of malignant effusions. Direct instillation of a radioactive colloidal gold suspension has been used widely, with improvement reported in 50 to 65 per cent of the patients.[3-10]

This method of therapy offers several advantages over external irradiation. The radioactive colloidal suspension spreads over the serosal surface, exposing a large area to irradiation. At the same time, the radiation is confined and selective so that there is less damage to normal tissue. Radiation sickness is not an appreciable factor, and the therapy is usually well tolerated. Since the radiation is administered in one treatment the discomfort produced by weeks of external irradiation is obviated.

There are, however, several disadvantages to the use of radioactive colloidal gold. Special facilities, as well as specially trained personnel, are required for its use. Since it has a short half life of 2.5 days, it is not always available, it is difficult to handle, and it is expensive to the patient. Radioactive gold must be ordered when therapy is decided upon and must be used almost immediately after it is received. Furthermore, it is a potential radiation hazard to the patient, doctors, nurses, and technicians. Routine use of radioactive colloidal gold is therefore not feasible in most hospitals.

The introduction of alkylating agents has made possible a simple, effective, universally available, and inexpensive method of therapy of malignant effusions.[11-19] This report describes the results obtained with the intracavitary instillation of methyl-bis(β-chloroethyl)amine hydrochloride (nitrogen mustard, HN2) in the management of malignant serous effusions.

Material and Methods

Eighty-eight patients with malignant serous effusions involving the pleural, pericardial, or peritoneal cavities have been treated during the past $3\frac{1}{2}$

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* The work reported in this paper was aided by a grant from the Cuyahoga Chapter of the American Cancer Society, Inc., Cleveland, Ohio, and by the Frederick Sands Memorial Fund.

years with a single dose of HN2 instilled directly into the involved cavity (TABLES 1 and 2). Twenty-four of these patients had carcinoma of the breast, 29 carcinoma of the ovary, 9 carcinoma of the lung, 5 gastrointestinal malignancies, and 11 malignant lymphomas, and the remainder had either an undiagnosed primary site or a variety of tumors such as mesothelioma and fibrosarcoma. Fifty-five patients had pleural effusions, 30 had peritoneal effusions, and 3 had pericardial effusions.

TABLE 1

INTRACAVITARY NITROGEN MUSTARD THERAPY

Site of primary malignancy	No.	Improved	Per cent improved
Breast	24	17	71
Ovary	29	20	69
Lymphatic system	11	5	49
Lung	9	5	55
Gastrointestinal	5	2	40
Miscellaneous	10	7	70
Total	88	56*	64% (Average)

*43—Complete remission; 13—partial remission.

All patients received premedication consisting of barbiturates and chlorpromazine. In patients with pleural or peritoneal effusions, most of the fluid was first removed to avoid excessive dilution of the alkylating agent. A single dose of HN2 was then instilled directly into the involved cavity; an adequate free flow was ensured by repeated aspiration of the effusion into the syringe during the period of injection. In most instances the dosage of HN2 employed was 0.4 mg. per kg. body weight. In patients with pericardial effusions, only 10 to 20 mg. of HN2 was administered. The results were compared with those obtained in a similar group of patients who had been treated with radioactive colloidal gold.

Results

Fifty-six of the 88 patients (64 per cent) treated by this method were significantly improved (TABLE 1). In 43 of these 56 patients there was no reaccumulation of fluid during their remaining course or up to the time of this report. In 13 patients, the amount and rate of fluid reaccumulation were significantly decreased. The best responses were usually obtained in patients with effusions due to metastatic carcinoma from the breast or ovary. All 3 patients with pericardial effusion had complete cessation of fluid formation. The results obtained with pleural effusions (67 per cent improvement) were somewhat better than those obtained with peritoneal effusions (53 per cent improvement) (TABLE 2).

Of the 32 failures, 6 occurred in patients with either chylous effusions or with effusions in which the specific gravity was less than 1.014. Seventeen of the patients who received no benefit from the direct instillation of HN2

TABLE 2
INTRACAVITARY NITROGEN MUSTARD THERAPY

Site of therapy	No.	Improved	Per cent improved
Pleura	55	37	67.5
Peritoneum	30	16	53.0
Pericardium	3	3	100.0
Total	88	56	64% (Average)

were in the terminal phases of their disease and died within 1 month (3 days to 4 weeks). Retreatment 1 month after an initial failure was successful in 3 patients.

The results obtained by the direct instillation of HN2 are comparable to those obtained in patients treated with intracavitary radioactive colloidal gold: 60 per cent of 60 patients who received therapy with colloidal radioactive gold at the University Hospitals of Cleveland were improved, compared to 64 per cent of 88 patients improved by intracavitary HN2 (TABLE 3).

TABLE 3
RESULTS OF INTRACAVITARY THERAPY: NITROGEN MUSTARD COMPARED WITH RADIOACTIVE COLLOIDAL GOLD

	Number treated	Number improved	Per cent improved
HN2	88	56	64
Au^{198}	60	37	60

The duration of benefit may be prolonged, depending upon the progress of the malignancy elsewhere in the body (FIGURE 1). Thus far, 14 of these patients have survived for periods ranging from 12 to 42 months, with complete relief of symptoms referable to their effusions. Some of the patients who have been treated during the past year may be expected to increase this number. Relapse at the site of effusion rarely occurs after successful therapy.

The side effects resulting from the intracavitary injection of HN2 are usually minimal. Pain resulting from the intrapleural administration of HN2 is uncommon and, when it does occur, it is usually transient, disappearing in 2 to 3 days. Pain following therapy for malignant peritoneal effusions is more common. The pain, however, it not severe, and it usually subsides in 2 to 3 days. With intrapericardial therapy, cardiac irregularities may occur, but these may be less troublesome than the cardiac tamponade usually associated with pericardial effusions. Nausea and vomiting are less severe than after intravenous administration of HN2, and in some instances may be completely obviated by adequate sedation and chlorpromazine.

In some patients a reactive pleural effusion with a temporary increase in symptoms may develop rapidly following therapy. This type of reaction usually is associated with a subsequent satisfactory result, but may necessitate a thoracentesis on the day following therapy. Following therapy an adhesive pleuritis with obliteration of the pleural space occasionally results. Since such obliteration prevents the reaccumulation of fluid, the patient obtains symptomatic relief and is also spared the necessity of frequent paracentesis. In most instances the serosal surface remains uninvolved, as shown by X-ray studies or post-mortem examination.

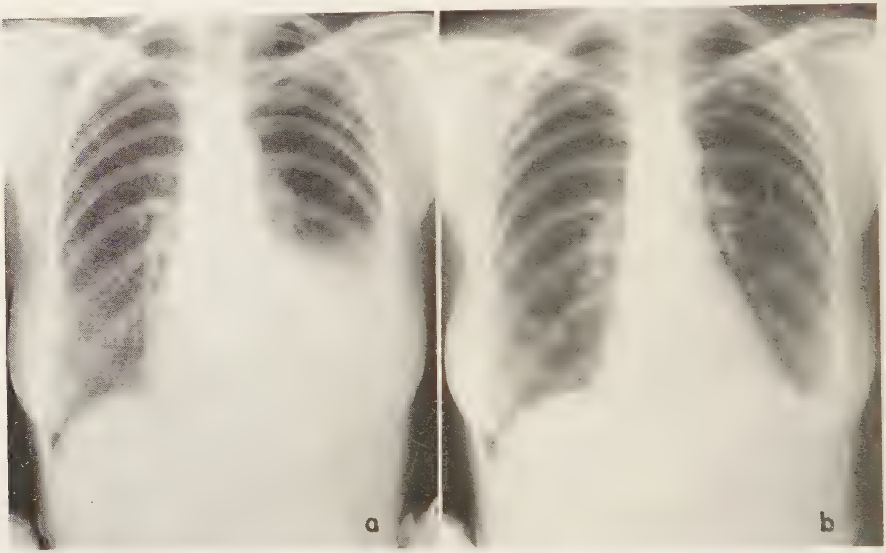

FIGURE 1. (*a*) Left pleural effusion in a woman with lymphoblastic lymphosarcoma before treatment; (*b*) same patient 3 years after intracavitary instillation of nitrogen mustard.

No adverse hematological effects were noted. The leukopenia following this type of therapy was very moderate and was much less than that occurring after intravenous HN2 therapy. No instances of thrombocytopenia or anemia attributable to intracavitary HN2 therapy occurred.

Comment

The direct instillation of HN2 into a malignant effusion offers the unique opportunity of placing an alkylating agent directly in contact with neoplastic cells. A high local concentration is achieved because dilution of the alkylating agent by the total blood volume is avoided. Furthermore, relatively prolonged contact is obtained because the HN2 is confined to the involved cavity. As a result, the local concentration and uptake of HN2 may be of sufficient magnitude to be cancerocidal in malignant tumors that ordinarily do not respond to HN2. Although intravenous therapy with HN2 is occasionally effective in treating certain types of malignant effusions it is usually ineffective in the therapy of many tumors that respond to the direct instillation of HN2.

Effusions resulting from mediastinal compression are unlikely to respond to intracavitary therapy. Thus, failures occur with chylous effusions, as well as with effusions that are low in specific gravity. Therapy is also more likely to be successful if it is instituted while the patient is still in good condition. Many failures in this series occurred in patients who were treated while in a terminal condition. In some, the duration of life after therapy was instituted was too brief to evaluate whether intracavitary HN2 might have been effective.

In view of the mildness of the leukopenia developing with this type of therapy, larger amounts of HN2 can probably be used without adverse side effects. Larger doses of HN2 may be effective in increasing the percentage of favorable responses, especially in peritoneal effusions, in which greater dilution is apt to occur and a larger area must be treated. Moreover, a second trial with intracavitary HN2 may be effective even if the first course of therapy results in failure. No instances of severe hematopoietic depression were encountered.

The favorable response following therapy is often more than temporary palliation. Long survivals with relatively good health can occur. If HN2 therapy is ineffective, a course of radioactive gold therapy is indicated. Such consecutive therapy is successful in a high percentage of cases. The reverse is also true: if radioactive gold is ineffective as initial therapy, the subsequent instillation of HN2 is frequently effective.

The results with intracavitary HN2 compare favorably with those obtained with radioactive colloidal gold. The use of HN2 has several advantages over radioactive colloidal gold. It is easier to administer, more universally available, and less expensive. It is relatively safe and presents no radiation hazards. At the present time, therefore, intracavitary HN2 appears to be the method of choice in the management of malignant effusions. Other alkylating agents may be equally effective or more effective than HN2.

Summary

The direct instillation of HN2 into malignant effusions offers a safe, fairly effective, inexpensive, universally available, and easily administered form of therapy. The results obtained are comparable to those obtained with radioactive colloidal gold. Long periods of survival with relatively good health are possible following successful therapy with HN2. At the present time the intracavitary administration of an alkylating agent appears to be the treatment of choice for patients with malignant effusions.

References

1. Muller, J. H. 1949. Zur medizinisch-therapeutischen Verwendung der künstlichen Radioaktivität. Bull. schweiz. Akad. med. Wiss. **5**: 484.
2. Muller, J. H. 1950. Further development of treatment of peritoneal and pleural metastasis from ovarian carcinoma with radioactive gold. Gynaecologia. **129**: 289.
3. Kent, E. M. & C. Moses. 1951. Radioactive isotopes in palliative management of carcinomatosis of pleura. J. Thoracic Surg. **22**: 503.

4. Smithers, D. W. 1951. Some varied applications of radioactive isotopes to localization and treatment of tumors. Acta Radiol. **35**: 49–61.
5. King, E. R., D. W. Spicer, F. W. Douda, M. A. Bender & W. E. Noel. 1952. The use of radioactive colloidal gold (Au^{198}) in pleural effusions and ascites. Am. J. Roentgenol. Radium Therapy Nuclear Med. **68**: 413–420.
6. Clark, T. H. 1952. Radioactive colloidal gold (Au^{198}) in the treatment of neoplastic effusions. Quart. Bull. Northwestern Univ. Med. School. **26**: 98.
7. Rose, R. G., M. P. Osborn & W. B. Stevens. 1952. The intracavitary administration of radioactive gold. New Engl. J. Med. **247**: 663.
8. Gould, A. A., S. W. Root, H. D. Kerman & R. P. Bigelow. 1953. Intracavitary colloidal radio-gold in the treatment of effusions caused by malignant neoplasms. Ann. Surg. **137**: 375–381.
9. Storaasli, J. P., F. J. Bonte, D. P. King & H. L. Friedell. 1953. The use of radioactive colloidal gold in the treatment of serous effusions of neoplastic origin. Surg. Gynecol. Obstet. **96**: 707–710.
10. Seaman, W. B., A. I. Sherman & M. Bonebrake. 1953. Radioactive gold in the treatment of malignant effusions. J. Am. Med. Assoc. **153**: 630–633.
11. Andrews, G. A., S. W. Root, H. D. Kerman & R. P. Bigelow. 1953. Intracavitary colloidal radiogold in the treatment of effusions caused by malignant neoplasms. Ann. Surg. **137**: 375.
12. Kent, E. M., C. Moses, W. B. Ford, E. R. Kutz & R. S. George. 1954. Radioactive isotopes in management of carcinomatosis of serous body cavities. A.M.A. Arch. Internal Med. **94**: 334–340.
13. Wheeler, H. B., W. E. Jacques & T. W. Botsford. 1955. Experiences with the use of radioactive colloidal gold in the treatment of cancer. Ann. Surg. **141**: 208.
14. Karnofsky, D. A., W. H. Abelmann, L. F. Craver & J. H. Burchenal. 1948. The use of nitrogen mustards in the palliative treatment of carcinoma, with particular reference to bronchogenic carcinoma. Cancer. **1**: 643–656.
15. Skinner, E. F. & D. Carr. 1952. Palliative therapy of incurable intrathoracic malignancies. Am. Practitioner and Digest Treatment. **3**: 900–910.
16. Albertelli, J., J. Monaco, T. O'Connor & G. E. Bur. 1954. Nitrogen mustard in serosal cavities in treatment of advanced malignant tumors. Prensa méd. Argent. **41**: 201–205.
17. Bateman, J. C., B. Moulton & N. J. Larsen. 1955. Control of neoplastic effusion by phosphoramide chemotherapy. A.M.A. Arch. Internal Med. **95**: 713.
18. Weisberger, A. S., B. Levine & J. P. Storaasli. 1955. The use of nitrogen mustard in the treatment of serous effusions of neoplastic origin. J. Am. Med. Assoc. **159**: 1704.
19. Weisberger, A. S., F. J. Bonte & L. G. Suhrland. 1956. Management of malignant serous effusions. Geriatrics. **11**: 23–30.

THE INTRAPERITONEAL USE OF NITROGEN MUSTARD AT THE TIME OF OPERATION FOR CANCER*

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Introduction

During the past four or five years there has accumulated evidence indicating that malignant cells are dislodged or dislocated during operative procedures for cancer. For example, it is reported[1-4] that, following resection of the colon for carcinoma, recurrences at the suture line develop in 5 to 15 per cent of cases. Smith and his associates[5] have recently shown that washings from the wound in 101 major operations for carcinoma were positive in 34 per cent of cases and suspicious in another 18 per cent. In their follow-up of only slightly more than 2 years, Smith and his colleagues noted 16 local recurrences in 34 cases in which tumor cells showed in the washings. As might be expected, the recurrence rate was higher in the patients showing positive washings than in the patients having negative washings (33 per cent versus 10 per cent). These data suggest that washing the wound does not remove all the cells, and also that there are other malignant cells that cannot be demonstrated by the washing technique. Since the follow-up of these investigators covered only 2 years, more time must elapse before their figures can be considered conclusive. Perhaps of more importance with regard to the dissemination of cancer cells is the demonstration,[6] made in 1953, that cancer cells may be found in venous blood draining from the tumor. Fisher and Turnbull[7] have carried this further and have reported finding cancer cells in 32 per cent of 25 specimens studied. Turnbull[8] has also noted that in 36 specimens examined for cancer cells in venous blood draining from the tumor, malignant cells were found in 28 per cent when the tumor was handled as much as usual incident to resection; however, in 76 resection cases in which precautions were taken not to handle the growth in the colon, the incidence of positive cells dropped to 13 per cent.

Perhaps more dramatic are the data reported by Engel,[9] who noted that cancer cells were found in the blood draining from the tumor in 59 per cent of 107 patients examined. In this group of patients cancer cells were found in such venous blood in 35 per cent of patients with grade II tumors, in 78 per cent of patients with grade III tumors, and in 100 per cent of patients with grade IV tumors. We found cancer cells in the peripheral blood in 4.6 per cent of 65 patients with operable cancer, and in 50 per cent of 14 patients with inoperable cancer.

In view of the evidence reviewed above that cancer cells may be dislodged at the time of operation, the authors have conducted experiments, as pre-

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viously reported,[10,11] indicating that methyl-bis(β-chloroethyl)amine hydrochloride (nitrogen mustard, HN2) and triethylene thiophosphoramide (Thio-TEPA) will decrease the percentage of "takes" when given to Sprague Dawley female rats after injection of a suspension of Walker 256 tumor cells into a tributary of the portal vein. For example, in 48 control rats receiving 110,000 cancer cells into a branch of the portal vein, the incidence of takes was 91.7 per cent, compared to an incidence of 17.8 per cent in 45 rats injected similarly with the same number of cells, but receiving nitrogen mustard (0.5 mg./kg.) 1 minute later into the same vein. When rats injected similarly with cancer cells into the liver were treated by nitrogen mustard via the peritoneal cavity 1 hour after inoculation, the takes were 86.7 per cent in the controls and 41.3 per cent in the treated animals. When the nitrogen mustard was given via a systemic vein it was also effective, though not quite to the same degree (75.6 versus 35.7 per cent). When Thio-TEPA was given (2.0 mg. per kg.) in the same manner as above, the effect was more marked. When 220,000 cells were injected into the rat's liver (via the mesenteric vein) the efficiency of therapy with Thio-TEPA was definitely lessened, as it was with nitrogen mustard. Preliminary experiments indicate that if the nitrogen mustard is given 24 or 48 hours later, it is not significantly effective; neither is it effective if given 1 hour before injection of the cancer cells.

In view of the favorable effects of nitrogen mustard and Thio-TEPA in preventing takes after inoculation of cancer cells into the liver, we initiated in March 1956 the clinical use of nitrogen mustard at the time of resection of tumor of the breast, colon, rectum, and stomach.

To date we have injected nitrogen mustard at the time of resection of the carcinoma in 65 patients. In 36 of these, the tumor was intraperitoneal and the HN2 was injected into a branch of the portal vein or was left in the peritoneal cavity at the end of the operation, or both.

Dosage of Nitrogen Mustard for Intraperitoneal Use

The initial dose of nitrogen mustard was given either intraperitoneally or into a tributary of the portal vein (usually an omental vein) or both. Using the latter route, the HN2 was administered by direct venipuncture or by threading a small polyethylene catheter into an omental vein. When the entire dose was left in the peritoneal cavity, it was dissolved in 500 cc. physiological saline and deposited in the peritoneal cavity by means of a catheter leading down to the area of resection as the last peritoneal stitch was taken. In the series reported herein, we gave 0.1 mg. HN2 per kg. as the first dose on the day of operation. The remainder of the total dose of 0.4 mg./kg. body weight (maximum 7.5 mg. per dose) was administered through a systemic vein on 3 successive days postoperatively. In order to minimize the possibility of serious reaction resulting from injection in patients who were poor surgical risks, we arbitrarily set 70 as the maximum age for intraperitoneal therapy. Likewise, we limited the total dose for each injection to 7.5 mg. to prevent giving an excessive amount to obese patients.

Thirty patients who had a resection were given an average dose of 5.7 mg. intraperitoneally and 16.9 mg. intravenously on successive postoperative days for an average total dose of 22.6 mg. HN2 (TABLE 1). Six patients who underwent only celiotomy were given an average dose of 7.0 mg. intraperitoneally and 13.6 mg. intravenously on successive days for an average total dose of 20.6 mg. HN2.*

Table 1
Dosage Schedule for HN2 Given as Adjuvant Therapy at Time of Operation
First dose on day of surgery I.P., remaining 2 or 3 doses I.V.

Type of case	No. of cases	Av. I.P. dose	Av. total I.V. dose	Total dose
Resection..............	30	5.7 mg.	16.9 mg.	22.6 mg.
Celiotomy only.........	6	7.0 mg.	13.6 mg.	20.6 mg.

Complications Following Use of Nitrogen Mustard Intraperitoneally

Since alkylating agents produce a sharp depression of the bone marrow, we suspected that because of a possible defect in the clotting mechanism and a decreased resistance to infection, nitrogen mustard would be more toxic when given at the time of operation than at other times. For this reason we started these injections cautiously.

Herein we have summarized our experiences with the 36 cases, including particularly the data on toxicity and complications. The drug has been given to 25 other patients at the time of operation (mostly for cancer of the breast), but in this report we shall present data on the intraperitoneal use only. Such cases were limited to operation for carcinoma of the colon, rectum, and stomach. The vast majority of these had a resection of their tumor.

Bone Marrow Depression

Almost all of the patients in this series developed a transitory leukopenia; that is, WBC below 5000 cu. mm. (TABLE 2). In 4 it fell to 3000 cu. mm. or less. Although several patients required supplemental transfusions, in all instances the leukocyte count returned to preoperative levels in 12 to 18 days.

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* For example, Anthony Curreri has informed us that he has given a large dose of HN2 intraperitoneally to 2 patients on the day of operation without ill effect. One of these patients had 22 mg. left in the peritoneal cavity after a colectomy for cancer; in the other, who had a gastrectomy for cancer, 30 mg. was deposited intraperitoneally at the time of surgery. W. Mengert informed us that he has given 30 mg. HN2 as 1 dose intraperitoneally to 4 cases without ill effect. In 2 of these there was a panhysterectomy for cancer and in the other 2 there was performed only a celiotomy because of inoperable cancer. Mengert gave 45 mg. intraperitoneally to another patient at the end of a panhysterectomy for cancer. This patient died on the ninth postoperative day with a lower nephron nephrosis syndrome. The dose given her (0.6 mg./kg. body wt.) is larger than any we have given.

There was a slight but definite tendency for the wounds of a few of the treated cases to bleed more than usual at the time of surgery. Two perineal wounds bled much more than usual for a few days postoperatively. For this reason, in the cases treated with HN2 and in their controls, we tabulated the blood requirements for the preoperative and for the operative and subsequent hospital periods. For the preoperative period there was no significant difference between the two groups (TABLE 3). A study of the blood requirements for the period encompassing surgery and the postoperative convalescence revealed the fact that the patients treated wth HN2 required an average of 1657 cc. as compared with 739 cc. for the control group. Actually, most of this difference is related to bone marrow depression rather than actual blood loss; most of the transfusions were given in the latter part of operative convalescence to combat a falling hematacrit incident to bone marrow depression.

TABLE 2

COMPLICATIONS WITH HN2 GIVEN AS ADJUVANT THERAPY—
C. Branch, 6 cases; W. Eastman, 2 cases; and A. Curreri, 2 cases

Type of operation	No. of cases	Complications			
		Leukopenia (−3000)	Bleeding	Ileus	Infection or obstruction
Colectomy	23	4	3	1	1
Gastrectomy	2	0	0	0	0
Miscellaneous	5	0	0	0	0
Celiotomy only	6	0	1	1	0
Total	36	4	4	2	1

One death 11 days after operation. Patient was 64 years old, white, female, having abdominoperineal resection. She had 6.5 mg. HN2 intraperitoneally, but none thereafter. Profuse diarrhea, electrolyte imbalance. Lowest WBC 6750.

TABLE 3

COMPARISON OF BLOOD REQUIREMENTS IN TREATED AND CONTROL CASES

	Pre-op.	Post-op.
HN2-treated	630 cc.	1657 cc.
Control	650 cc.	739 cc.
Difference	−20 cc.	918 cc.

Liver Dysfunction

Tests of liver function (thymol turbidity, Bromsulphalein (BSP), prothrombin time, alkaline phosphatase, and total protein with A/G ratio) were performed in practically all cases. A significant impairment was seen in only 1 patient whose postoperative BSP retention was 35.5 per cent in 45 minutes. This patient had a transfusion reaction that might have been the cause of this abnormal retention. A second patient had a 17.4 per cent BSP retention on the sixth postoperative day; when repeated a week later it was normal. In no patient was there any residual hepatic dysfunction attributable to HN2 therapy.

Infection

In one patient who received HN2 intraperitoneally at the conclusion of a sigmoid colectomy, a presacral abscess developed. This patient's white count never descended to leukopenic levels, and he recovered without further incident. In spite of the variable degree and duration of leukopenia in many of the treated cases in this series, postoperative infection was not a hazard.

Mortality

Of the 36 patients in this study, there was one postoperative death. This patient had an abdominoperineal resection, and received 6.5 mg. HN2 intraperitoneally only at the time of surgery. Because the patient appeared quite ill on the day following surgery, she was not given any more HN2. She developed uncontrollable diarrhea and it was difficult to keep her in electrolyte balance. Culture of the stool revealed a staphylococcus. It was our impression that this complication was much more readily attributable to chemotherapy than to the nitrogen mustard. The lowest WBC in this patient was 6750/cu. mm.

Miscellaneous Complications

Postoperative ileus was exceptional, appearing in only 2 cases. In 1, it persisted for 8 days, after which there was recovery without residual difficulty.

A patient who received only one dose (7.5 mg. HN2 intraperitoneally) after celiotomy for a nonresectable epidermoid carcinoma of the anus had a respiratory arrest and vascular collapse 30 minutes after termination of the operation. She was not given HN2 on succeeding days because the cause of the reaction could not be determined. It was concluded that this was an anesthetic complication, since she had received a fairly large dose of succinyl choline during the operation.

Summary

It is our opinion that, in view of the aforementioned experimental data, the intraperitoneal instillation of HN2 at the conclusion of a curative operation for cancer of the stomach, colon, or rectum is a safe procedure, providing certain precautions are utilized. We have adopted the principle of not giving HN2 to patients over 70 years of age, and have utilized 7.5 mg. as the maximum daily dose to prevent overdosage in obese patients. Utilizing these precautions, we do not believe the mortality rate will be significantly higher than in a control group, although we admit that in the control group of patients we had no deaths (this is unusual, since the mortality rate in resection for carcinoma of this type should be somewhere between 2 and 5 per cent). We expected complications to be higher in our treated cases, and they were. It should be noted, however, that in this series complications in our control group were unusually slight and that there were no serious complications. The most prominent difference in complications between the treated and control cases was a much greater need for blood postoperatively in the treated cases; most of this need was related to bone marrow depression.

References

1. Morgan, C. N. & O. V. Lloyd-Davies. 1950. Discussion on conservative resection in carcinoma of the rectum. Proc. Roy. Soc. Med. **43**: 701.

2a. Cole, W. H. 1951. Measures to combat the menace of cancer. Am. Surgeon. **17**: 660.

2b. Cole, W. H. 1952. Recurrence in carcinoma of the colon and proximal rectum following resection for carcinomas. Arch. Surg. **65**: 264.

3. Goligher, J. C., C. E. Dukes & H. J. R. Bussey. 1951. Local recurrences after sphincter-saving excision for carcinoma of the rectum and rectosigmoid. Brit. J. Surg. **39**: 199.

4. Ackerman, L. 1955. The implantation of cancer—an avoidable surgical risk? Surgery. **37**: 341.

5. Hilberg, A. W., R. R. Smith, R. M. Miller, R. V. Eck, A. G. Shih & W. Kramer. 1956. Wound seeding as a cause of failures in surgical therapy of cancer. Proc. Natl. Cancer Conf. 3rd Conf. 568.

6. Cole, W. H., D. Packard & H. W. Southwick. 1954. Carcinomas of the colon with special reference to prevention of recurrence. J. Am. Med. Assoc. **155**: 1549.

7. Fisher, D. R. & R. B. Turnbull, Jr. 1955. The cytologic demonstration and significance of tumor cells in the mesenteric venous blood in patients with colorectal carcinomas. Surg. Gynecol. Obstet. **100**: 102.

8. Turnbull, R. B. Personal communication.

9. Engel, H. C. 1955. Cancer cells in the circulating blood. Acta Chir. Scand. Suppl. No. **201**.

10. Cruz, E. P., G. O. McDonald & W. H. Cole. 1956. Prophylactic treatment of cancer. Surgery. **40**: 291.

11. McDonald, G. O., C. E. Livingston, C. F. Boyles & W. H. Cole. 1957. The prophylactic treatment of malignant disease with nitrogen mustard and Thio-TEPA. Ann. Surg. **145**: 624.

EARLY NITROGEN MUSTARD DERIVATIVES

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From 1946 to 1948, 5 different nitrogen mustard derivatives were studied clinically at the Memorial Center for Cancer and Allied Diseases on the basis of their activity against transplanted tumors and leukemias in mice. The first of these, 1,3-bis[bis(2-chloroethyl)amino]propane dihydrochloride (SK 136) was given to 34 patients, including 14 adults with chronic granulocytic leukemia and 13 children with acute leukemia.

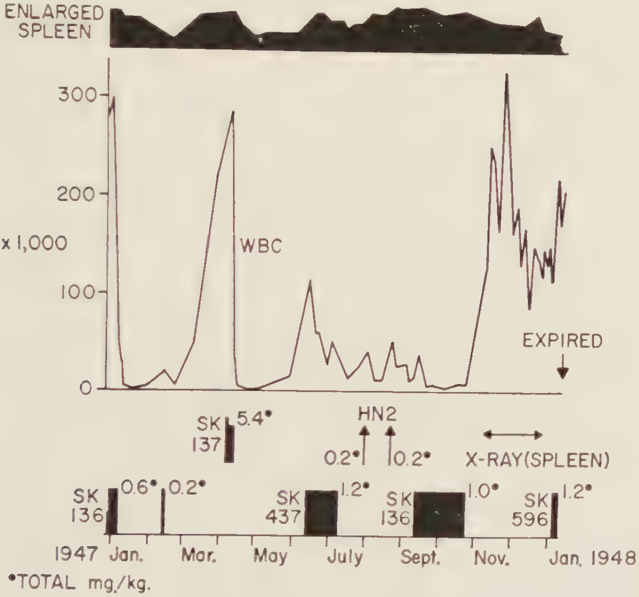

FIGURE 1. H. F. 43-year-old man. Chronic granulocytic leukemia.

When SK 136 was given intravenously at 0.1 mg. kg. per day, with the total course given in 4 to 8 daily injections, there was almost no nausea and vomiting, in contrast to methyl-bis(β-chloroethyl)amine (HN2), but a few of the patients noted some anxiety during the course of treatment, and one patient reported a true hallucination, lasting for 2 or 3 hours after injection. As with the nitrogen mustard HN2, this compound was active against the chronic leukemias (FIGURE 1) and the lymphomas. It did not appear, however, to have any therapeutic advantage over HN2 other than the slight decrease in nausea and vomiting.

A closely allied derivative, 1,3-bis[bis(2-chloroethyl)amino]2-chloropropane dihydrochloride (SK 137), at daily doses of 0.4 to 1.0 mg./kg. intravenously, was given to 16 patients, again with very little nausea and vomiting. However, among these patients there were 4 who developed definite agitation and temporary psychoses and, for this reason, further evaluation of the drug was discontinued, despite the fact that it seemed to show therapeutic activity approximately equal to HN2 (FIGURE 1).

The methoxypyridoxine analogue of HN2, known as SK 1424, produced dizziness, nausea and vomiting, and disturbances in taste effects that made it difficult to administer in effective therapeutic doses. 1,2,3,4-Tetrakis-[bis(2-chloroethyl)amino]butane tetrahydrochloride (SK 596) and methyl-bis(β-bromoethyl)amine hydrobromide (SK 437) were given intravenously to 5 and 4 patients, respectively. In 3 patients with chronic granulocytic leukemia, SK 437 at a dose of 0.1 mg./kg. daily for 7 to 12 days produced satisfactory falls in total leukocyte count, decrease in spleen size, and subjective improvement (FIGURE 1). In one patient with acute leukemia no beneficial effect was noted. Nausea and vomiting occurred after injection, as with HN2. SK 596 similarly caused lowering of total leukocyte count and decrease of spleen size in a patient with chronic granulocytic leukemia at an average dose of 1.0 mg./kg. for 10 to 15 days (FIGURE 2), but caused dizziness, nausea, and occasional vomiting in 2 of 5 patients. Although both of these compounds showed clinical evidence of activity against chronic leukemias, there was no indication that they possessed any advantages over HN2.

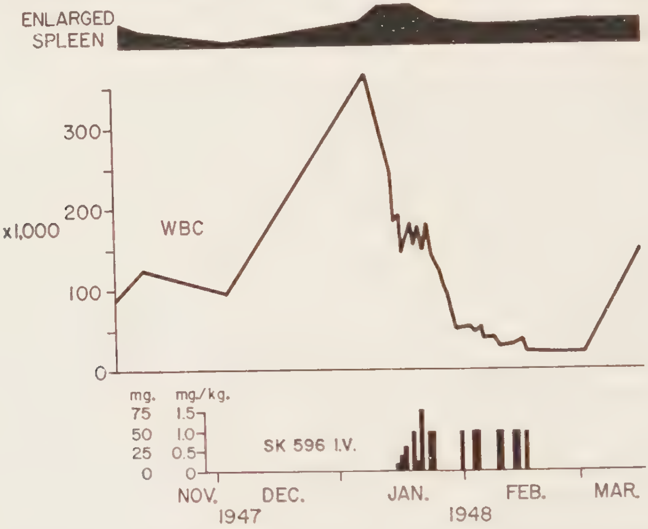

FIGURE 2. M. B. 62-year-old woman. Chronic granulocytic leukemia.

Summary

Certain early derivatives of the nitrogen mustards at therapeutic dos levels produced less nausea and vomiting than HN2, but the increased neurotoxicity prevented them from being practical agents. None of the 5 agent studied possessed any practical therapeutic advantage over HN2.

CLINICAL EXPERIENCE WITH NITROMIN

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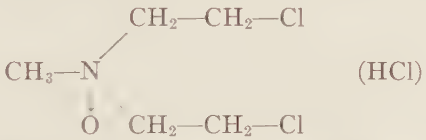
This paper deals with our clinical experiences with Nitromin in the treatment of tumors. Among the various alkylating agents, Nitromin, triethylene melamine (TEM), and triethylene thiophosphoramide (thio-TEPA) are in use in Japan. Of these, Nitromin is at present the first choice of Japanese doctors.

We had hoped to collect clinical data on Nitromin from all Japan, but the time was too short to do so. Therefore, this presentation will be restricted to clinical experiences with Nitromin in the Tokyo First National Hospital and to data from reports at meetings of the Cancer Treatment Committee of the Health and Welfare Ministry of the Japanese government. In addition we shall present a comparison of Nitromin with other alkylating agents.

Structure and Source

Nitromin, methyl-bis(β-chloroethyl)amine-*N*-oxide hydrochloride, has the following formula:



$$\begin{array}{l} \qquad\quad CH_2-CH_2-Cl \\ CH_3-N\diagup \qquad\qquad\qquad\qquad (HCl) \\ \qquad\ \downarrow\diagdown \\ \qquad\ O \quad CH_2-CH_2-Cl \end{array}$$

This substance was reported in a series of chemical warfare agents by Stahmann and Bergmann[1] in 1946. Morizo Ishidate[2] and Tomizo Yoshida of the University of Tokyo found the substance active in their study on growth-retarding agents against the Yoshida sarcoma. This compound was obtained by the oxidation of nitrogen mustard with hydrogen peroxide in the presence of an organic anhydride.

Purity and Stability

The hydrochloride of this drug occurs as prismatic, odorless white crystals with a melting point of 109° to 110° C., and is readily soluble in water and in alcohol. The *p*H value of the hydrochloride is estimated as 2.8 for a 0.052 molar solution, while that of nitrogen mustard in the same concentration is 3.4. Since it has a higher stability than nitrogen mustard, no change is discernible in a 0.5 per cent solution even if left for 24 hours at 15° C.

Route of Administration and Usual Dosage

Although intravenous injection is in common use, intra-arterial injection and local and oral administration are feasible. Dosages are shown in TABLE 1.

The intervals for administration should be every day or every other day, and once or twice a week for intrathoracic or intraperitoneal administration. The usual total dosage in one course is 500 mg. The maximal total dosages were 2600 mg. by vein, 6000 mg. by artery, and 2800 mg. by mouth.

Table 1

Route of Administration and Usual Dosage

Route of administration	Usual dosage/day
Intravenous injection	0.5–1.0 mg./kg. body weight
Oral administration	0.5–1.0 mg./kg. body weight
Intra-arterial injection	0.05–0.1 mg./kg. body weight
Intrathoracic or intraperitoneal administration	0.5–1.0 mg./kg. body weight
Local administration	Physiological saline solution of 0.1–1.0 mg./cc. density

The drug was administered intravenously and orally by dissolving it in a 20 per cent glucose solution or in a physiological saline solution of 1.25 mg./cc. density, and intra-arterially by giving a solution of 0.0125 mg./cc. density through a polyethylene tube.

No thrombosis resulted from the use of these methods.

Toxicity in Man

The toxicity of Nitromin in 167 cases is shown in table 2, the data for which are derived from the results obtained by the Cancer Treatment Committee.

Table 2

Toxicity of Nitromin in Man

(167 cases)

Side effects	Number of cases	%	Side effects	Number of cases
Leukopenia	67	39	Fever	2
Loss of appetite	63	38	Perspiration	1
Nausea	52	31	Exanthema	2
Vomiting	28	17	Dyspnea	1
Headache	15	9	Palpitation	1
Fatigue	11	6	Cyanosis	1
Abdominal pain	6	4	Arrhythmia	1
Transient mental disturbance	3	2	Chest pain	1
Chill	2			

In these cases, such symptoms as loss of appetite, nausea, vomiting, headache, fatigue, and abdominal pain were observed, as were chills accompanied by fever and perspiration. Exanthema, transient mental disturbance, dyspnea, cyanosis, palpitation, arrhythmia, chest pain, and paresthesia occurred in a few cases. Liver and kidney functions were unaffected.

With regard to leukopenia and bone marrow depression the following tendencies were observed: in peripheral blood, slight anemia, leukopenia, and slight thrombocytopenia occurred, but there was no hemorrhagic diathesis. At the time of maximal leukopenia in peripheral blood, the numbers of bone marrow cells decreased in half of the cases, but not drastically. Granulocytopoiesis, especially in immature cells, was slightly depressed, as shown by the myelogram, and the peripheral leukopenia came mainly from neutropenia. Lymphocytopoiesis was also depressed, but erythropoiesis was not influenced as much as granulocytopoiesis.[3]

This leukopenia in peripheral blood usually recovers within two or three weeks after treatment, but when we repeated the Nitromin treatment, the recovery from leukopenia was prolonged.

TABLE 3
NUMBER AND TYPES OF PATIENTS

Far-advanced and inoperable cancer		124
Carcinoma of stomach and esophagus	34	
Carcinoma of liver	9	
Carcinoma of pancreas	6	
Carcinoma of lung	37	
Mammary carcinoma	8	
Choriocarcinoma	12	
Other types	18	
Sarcoma		19
Reticulosarcoma	9	
Lymphosarcoma	1	
Other types	9	
Leukemia and allied diseases		51
Acute leukemia	23	
Chronic myelogenous leukemia	23	
Hodgkin's disease	4	
Multiple myeloma	1	
Other tumors		35
Tumor of testis and ovary	6	
Tumor of brain and spinal cord	21	
Other malignant tumors	8	
Total		229

In addition, there was depression of spermatogenesis. According to Nakamura[4] of Shimizu's surgical clinic of the Medical School of the University of Tokyo, the number and motility of the spermatozoa decreased, and malformed spermatozoa appeared. The depressed spermatogenesis recovered much later than did the leukopenia.

Number and Types of Patients

Two hundred and twenty-nine patients were treated with the drug: 124 cases of far-advanced and inoperable cancer; 19 of sarcoma; 51 of leukemia and allied diseases; and 35 other cases (TABLE 3).

All were admitted during the last five years to five hospitals three university hospitals and two national hospitals. All these hospitals were members of the Cancer Treatment Committee of the Health and Welfare Ministry of the Japanese government.

The drug was also given to 167 additional cases after surgical treatment for gastric cancer.

Summary of Clinical Results

Objective improvement through the use of Nitromin was observed in chronic myelogenous leukemia (15 of 23), lymphosarcoma (1), choriocarcinoma (8 of 12), mammary carcinoma (4 of 8), lung cancer (9 of 37), cancer of the liver (1 of 9) and tumor of the testis or ovary (2 of 6). The results are shown in TABLE 4.

TABLE 4
SUMMARY OF CLINICAL RESULTS IN THE TREATMENT OF TUMORS WITH NITROMIN

Types of tumors	Number of cases	Objective improvement	
		Number of cases	Per cent
Carcinoma			
Stomach and esophagus	34	3	9
Liver	9	1	11
Pancreas	6	1	17
Lung	37	9	24
Mammary gland	8	4	50
Choriocarcinoma	12	8	75
Other types	18	4	22
Total	124	30	24
Sarcoma			
Reticulosarcoma	9	6	67
Lymphosarcoma	1	1	
Other types	9	1	11
Total	19	8	42
Leukemia and allied diseases			
Acute leukemia	23	3	13
Chronic myelogenous leukemia	23	15	65
Multiple myeloma	1	0	0
Hodgkin's disease	4	1	25
Total	51	19	37
Other tumors			
Tumor of testis and ovary	6	2	33
Tumor of brain and spinal cord	21	3	14
Other malignant tumors	8	1	17
Total	229	63	27.5

The objective improvements in each case are as follows. In chronic myelogenous leukemia, immature cells were decreased according to the fall of the leukocyte count in the peripheral blood, and a more normal distribution

Table 5
Survival Rate in Nonscirrhous Cancer after Gastrectomy
Without Nitromin

Years	Number of cases	Survival time (months)			
		6	12	24	36
1952	13	10	9	6	4
1953	8	7	6	2	
1954	24	19	11		
1955	18	10			
Total..........	63	46	26	8	4
Per cent.		73.0	57.7	38.0	33.0
With Nitromin (500 mg.)					
1952	2	2	2	2	2
1953	15	13	9	7	
1954	21	19	17		
1955	14	12			
Total..........	52	46	28	9	2
Per cent........		88.5	73.6	52.9	100

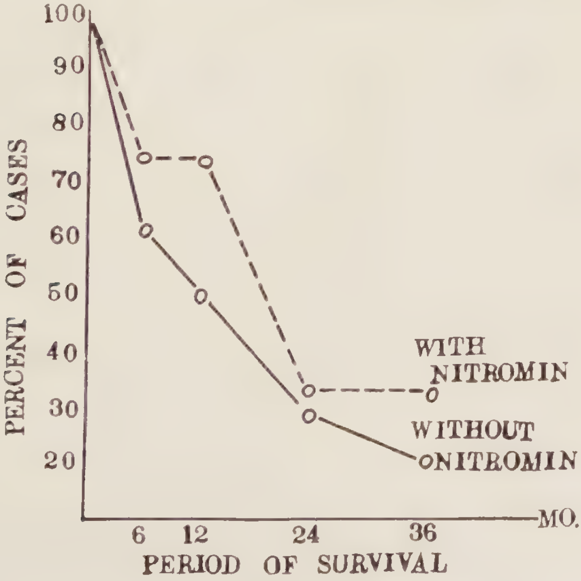


Figure 1. Partial gastrectomy. Scirrhous cancer.

Table 6
Survival Rate in Scirrhous Cancer after Gastrectomy
Without Nitromin

Years	Number of cases	Survival time (months)			
		6	12	24	36
1952	5	3	2	1	1
1953	2	2	2	1	
1954	9	4	4		
1955	2	2			
Total.........	18	11	8	2	1
Per cent.......		61.1	50.0	28.5	20
With Nitromin (500 mg.)					
1952	3	2	1	1	1
1953	6	4	4	2	
1954	6	6	6		
1955	7	4			
Total.........	22	16	11	3	1
Per cent.......		72.7	73.3	33	33

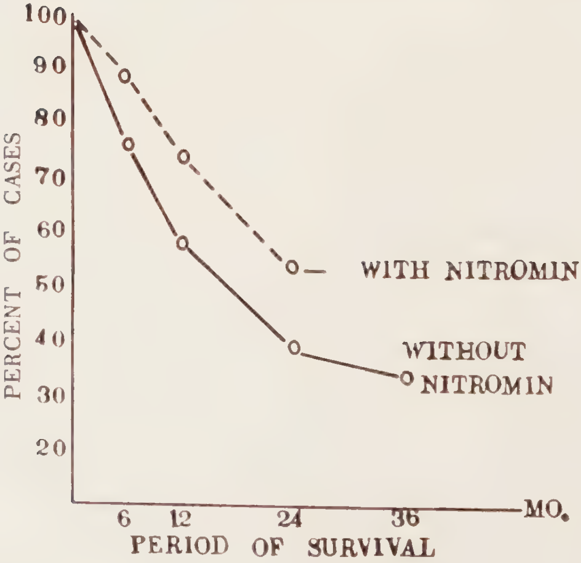


Figure 2. Partial gastrectomy. Nonscirrhous cancer.

occurred in the myelogram. The enlargement of the spleen and liver was reduced in the course of the treatment. When the bone marrow was markedly improved, we found excellent remission.

In Hodgkin's disease, reticulosarcoma, and lymphosarcoma, the fever fell and enlargement of the lymph nodes, spleen, and liver diminished. In choriocarcinoma reduction of the tumor and disappearance of the positive pregnancy test were observed. In bone-destroying metastases of mammary cancer we found signs of new bone formation. In lung cancer X-ray films revealed that the abnormal shadow had disappeared or had become markedly smaller. In the case of large tumors of the liver, testis, and ovary some diminished and others disappeared.

Encouraging results are being obtained from the use of Nitromin in the postoperative treatment of gastric cancer. Nitromin was given within 2 to 3 weeks after surgery in a total dosage of 500 mg. in each case.

More promising results have been obtained thus far in nonscirrhous types of cancer than in those of scirrhous types[5] (TABLES 5 and 6; FIGURES 1 and 2).

Relationship Between Therapeutic Response and Degree of Depression of Peripheral Blood Count

It is said that the objective improvements produced by treatment with alkylating agents, especially with nitrogen mustard, are unavoidably accompanied by significant leukopenia. However, in our 51 patients who received

TABLE 7

RELATIONSHIP BETWEEN THERAPEUTIC RESPONSE AND DEGREE OF DEPRESSION OF PERIPHERAL LEUKOCYTE COUNT

Objective improvements	Leukopenia*	Unchanged	Total
Present	16	14	30
None	14	7	21
Total	30	21	51

* Decrease of peripheral leukocyte count was more than ⅓ of that measured before treatment.

Objective improvements	Leukopenia†	Unchanged	Total
Present	9	21	30
None	9	12	21
Total	18	33	51

† Decrease of peripheral leukocyte count was more than ½ of that measured before treatment.

TABLE 7 shows no statistical significance.

a total of more than 250 mg. Nitromin, no statistically significant relation between objective improvements and leukocyte count in the peripheral blood has been established; that is to say, in many cases treated with Nitromin the significant improvements were present without any change of leukocyte count (TABLE 7).

Response of Patients to Other Alkylating Agents

We compared the clinical results obtained by the use of Nitromin with those from nitrogen mustard, triethylene melamine (TEM), and triethylene

TABLE 8
COMPARISON OF CLINICAL RESULTS (OBJECTIVE IMPROVEMENT) WITH VARIOUS ALKYLATING AGENTS

Names of drugs	Nitromin		Nitrogen mustard		TEM		Thio-TEPA	
Types of tumors	No. of cases	%	No. of cases	%	No. of cases	%	No. of cases	%
Carcinoma								
Stomach and esophagus	3/34	9	1/1		0/2		1/13	7
Liver	1/9	11	0		1/1		2/2	
Pancreas	1/6	17	0/1		0		1/1	
Lung	9/37	24	3/4	75	0/6	0	5/11	46
Mammary gland	4/8	50	0		0		9/21	43
Choriocarcinoma	8/12	75	0		0		0/1	
Others	4/18	22	1/1		0		0/5	
Total	30/124	24	4/9	44	1/9	11	18/54	33
Sarcoma								
Reticulosarcoma	6/9	67	1/1		0/2		5/12	43
Lymphosarcoma	1/1				2/3		0/1	
Others	1/9	11			2/5	40	0/1	
Total	8/19	42	1/1		4/10	40	6/14	43
Leukemia and allied diseases								
Acute leukemia	3/23	13	3/11	27	5/12	42	2/3	
Chronic leukemia	15/23	65	4/6	66	22/32	69	6/8	75
Myeloma	0/1		0		0		1/1	
Hodgkin's disease	1/4	25	0		2/3		2/3	
Total	19/51	37	7/17	41	29/47	62	11/15	73
Other tumors								
Tumor of testis and ovary	2/6	33	0		0		0/1	
Tumor of brain and spinal cord	3/21	14	0		0		0	
Others	1/8	17	0		0		2/3	
Total	229		27		61		87	

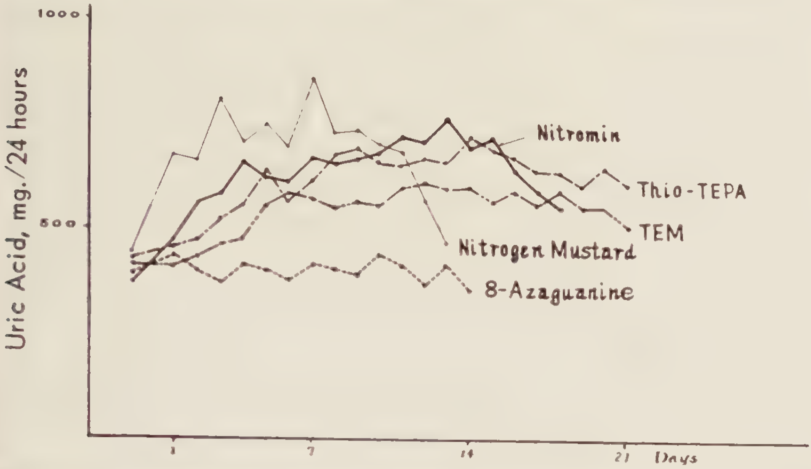


FIGURE 3. Comparison of the amount of daily urinary excretion of uric acid during the treatment of cancer patients with alkylating agents.

TABLE 9
COMPARISON OF SIDE EFFECTS WITH ALKYLATING AGENTS

Names of drugs	Nitromin		Nitrogen mustard		TEM		Thio-TEPA	
Side effects	No. of cases	%	No. of cases	%	No. of cases	%	No. of cases	%
Loss of appetite	36	44	7	63	9	43	6	13
Nausea	36	44	7	63	6	28	7	15
Vomiting	21	26	2	18	3	14	4	9
Pain	10	12	4	36	1	5	1	2
Fatigue	8	9	0	0	0	0	0	0
Fever	3	4	3	27	1	5	0	0
Chill	2	2	5	45	0	0	0	0
Cyanosis	2	2	1	9	0	0	0	0
Dyspnea	1	1	1	9	0	0	0	0
Exanthema	2	2	0	0	1	5	0	0
Leukopenia	18/58	31	5/10	50	6/19	32	10	22
Thrombocytopenia	4/15	26	—	—	2/11	18	9/38	23
Hemorrhagic diathesis	0	0	0	0	4	19	2	4
Total number of cases	81		11		21		45	

hiophosphoramide (Thio-TEPA) in the treatment of malignant diseases TABLE 8). In chronic leukemia we found no difference among these four rugs, but in acute leukemia Nitromin was less effective than TEM and Thio-TEPA. Nitromin was rather advantageous in reticulosarcoma, but no ifference was observed in sarcoma as a whole. In the treatment of carcinoma there was no significant difference among the four drugs, but the effect of nitrogen mustard on lung cancer was remarkable.

The daily excretion of uric acid in the urine during the treatment of cancer varies, depending on the drug administered: it is largest with nitrogen mustard, less with Nitromin, still less with Thio-TEPA, and least with TEM (FIGURE 3).

As for the side effects, there were no remarkable differences among Nitromin, TEM, and Thio-TEPA. Nitrogen mustard, however, caused more severe symptoms than the other three drugs, both in bone marrow depression and in gastrointestinal symptoms. Loss of appetite, nausea, and vomiting appeared less frequently with Thio-TEPA than with the other two drugs (TABLE 9).

The incidence of leukopenia and thrombocytopenia was almost the same with Nitromin, TEM, and Thio-TEPA. We found hemorrhagic diathesis with the latter two drugs, but not with Nitromin; we consider this to be an important factor in the treatment of outpatients.

Summary

The structure, source, purity, stability, toxicity in man, route of administration, usual dosage, and summary of clinical results with Nitromin have been presented. The advantages and weak points of Nitromin have been compared with those of nitrogen mustard, TEM, and Thio-TEPA.

According to our clinical experiences, these four drugs fall into two groups: Thio-TEPA and Nitromin; and nitrogen mustard and TEM. For clinical use the former group is preferable to the latter, which has many side effects.

The advantages of Nitromin are: stability, minimal local damage, no thrombosis, and significant improvement without leukopenia.

With regard to side effects, Thio-TEPA produced the least symptoms in the gastrointestinal tract. However, it caused hemorrhagic diathesis while Nitromin did not.

The results presented here were obtained by the use of larger amounts of the drug (1.0 mg. kg. body weight) than the usual present dosage (0.5 mg./kg.). In the near future we hope to reduce the side effects of the drug remarkably, while attaining the same clinical results.

References

1. STAHMANN, M. A. & M. BERGMANN. 1946. Chemical reactions of the nitrogen mustard gases. VIII. The oxidation of the nitrogen mustard gases by peracids. J. Org. Chem. **11**: 586.
2. ISHIDATE, M., K. KOBAYASHI, Y. SAKURAI, H. SATO & T. YOSHIDA. 1951. Experimental studies on chemotherapy of malignant growth employing Yoshida sarcoma animals. II. The effect of N-oxide derivatives of nitrogen mustard. Proc. Japan Acad. **27**: 493 and addendum.
3. KANNO, M., N. UTIMI, S. SATO, T. YUKI & K. ICHIKAWA. 1954. Influence of nitrogen mustard N oxide upon the blood picture and bone marrow. Tôhoku Igaku Zasshi. **49**: 165.
4. NAKAMURA, K. 1954. Studies of the influence of Nitromin on the spermatogenesis. Gekanoryoiki. **2**: 272, 329.

5. Ohara, T., H. Tokuyama & J. Tokuoka. 1957. Long term observation of gastric cancer treated by surgery and intravenous Nitromin. I. Japan. J. Cancer Clin. **3**: 554.
6. Otsuki, K. 1956. Present status of the treatment of cancer (malignant neoplasma). Geka. **18**: 601.
7. Ishizuka, N., T. Ide, M. Matuyama, Y. Okuda, M. Mashimo & H. Kashima. 1953. The treatment of chorioepithelioma chemotherapy. Sanka to Fuzinka. **20**: 439, 519.
8. Koyama, Y. 1956. Chemotherapy of malignant neoplasm (2). Gan no Rinsho. **2**: 150.

CLINICAL EXPERIENCE WITH THE USE OF R48 (N,N-DI-2′-CHLOROETHYL-2′-NAPHTHYLAMINE)

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As an extension of the observed cytotoxic action of aliphatic 2-chloroethylamines, Ross[1] prepared for biological testing a large number of haloalkylarylamines. In 1948 Haddow *et al.*[2] reported the effects upon tumors of various members of this series. They noted that marked cytotoxic and inhibitory activity, as judged against the growth of various animal tumors, was a feature of the N,N-di-2-chloroethyl derivatives of aniline, *o*-, *m*- and *p*-toluidine, *o*- and *p*-anisidine, β-naphthylamine, and *p*-aminobenzoic acid.

One of these, N,N-di-2′-chloroethyl-2′-naphthylamine (R48, now known as CB 1048), showed less toxicity than others and, in a dosage of 70 to 100 mg./kg. administered by single injection into the peritoneal cavity of the rat the day following implantation with the Walker tumor, was observed to inhibit development of the graft in about half the animals and to extend the survival period considerably in the remainder.

Supplies of this material for clinical trial were made available to us by Haddow, who has informed us that it was prepared by the Boots Pure Drug Company, Ltd., Nottingham, England, and purified in the laboratories of the Chester Beatty Research Institute, London. It is a pure organic compound.

This report covers the experience in the clinical use of R48 at the Royal Infirmary, Edinburgh, and at the Toronto General Hospital. It was only one of a number of chemotherapeutic agents then used in the management of patients with malignant lymphoma, leukemia, and allied disease, but in each of our clinics it was the first alkylating agent to be used orally.

R48 was usually given in doses of 50 to 400 mg. daily, the average dose being 100 to 200 mg. In a few cases, 25 mg. daily was sufficient for maintenance therapy.

In our experience R48 has been relatively nontoxic. Anorexia, nausea, vomiting, diarrhea, dysuria with frequency of urination, and skin rash have been encountered. When starting patients on treatment it was our custom to use doses of 200 mg. or more daily. Under these circumstances most patients experienced some anorexia and nausea and, in a few cases, vomiting and diarrhea. With subsequent decrease in dose these toxic effects usually disappeared. Dysuria and frequency of urination occurred in several patients who were receiving 400 mg. daily as a short course of treatment. In these patients microscopic hematuria was observed, but disappeared as soon as the dose was reduced to 200 mg. daily. An unusual complication was the occurrence of a maculo-erythematous rash. Cessation of therapy was followed by disappearance of the rash and, interestingly enough, the rash did not reappear when the agent was given again.

An important feature of R48 therapy is that the depressant effect on marrow and lymphocytes occurs slowly, an interval of two or three weeks occurring before any noticeable effect is observed. This is an advantage in the management of ambulatory patients since blood examinations need be done only at two-week intervals. Because of this slow action in the dosage used and because patients were observed at least once every two weeks while on treatment, marrow depression severe enough to cause trouble clinically was not observed.

Previous reports[3, 4, 5] have summarized the results of clinical trials with R48. Matthews,[3] in a total of 17 cases, reported partial remissions in Hodgkin's disease and in chronic myeloid leukemia. Satisfactory remissions were reported in 2 of 4 cases of chronic lymphatic leukemia and in 1 case of polycythemia rubra vera. Gardikas and Wilkinson,[4] in 25 cases, reported comparable results. Iversen and Meulengracht[5] reported favorable remissions in polycythemia.

We have used R48 in 92 cases, as shown in TABLE 1.

TABLE 1
CASES TREATED WITH R48

Disease	Number
Hodgkin's disease	13
Reticulum cell sarcoma	9
Mycosis fungoides	3
Giant follicle lymphoma	8
Lymphosarcoma	7
Acute leukemia	4
Chronic lymphatic leukemia	29
Chronic myeloid leukemia	7
Multiple myeloma	8
Miscellaneous carcinoma	4
Total	92

In each of our clinics radiotherapy is generally the main form of treatment for patients with malignant lymphoma, chronic leukemia, and multiple myeloma. Chemotherapy is used when radiotherapy is considered impracticable by reason of extent of disease, or is considered to be of no further value in the management of the individual case. Cases treated by chemotherapy are, therefore, a select group, in that almost all have had radiotherapy previously or are in the advanced stages of their disease.

In attempting to assess the clinical value of chemotherapeutic agents for the purpose of reporting we have adopted certain criteria. Unless symptoms and signs of improvement are definite and of at least three months' duration we do not consider that a satisfactory remission has been achieved. This is not to say, of course, that a remission of less extent is of no value clinically.

TABLE 2 summarizes the results of treatment as based on the above criteria. From this table it is apparent that the best results were observed in patients with chronic lymphatic leukemia and lymphosarcoma. We are quite familiar with the chronic and undulating course of these diseases, but feel that the cases that we classified as having a satisfactory remission did benefit materially from R48 therapy. Another point to be made is that patients with chronic lymphatic leukemia made up the largest single group. This is true for the following reason: often there is no urgency in treating this group, and we were content to use R48, having become familiar with its relatively slow action and the ease and safety with which it could be used in ambulatory patients. In patients in whom there was some urgency in obtaining relief of symptoms we preferred to use bis β-chloroethylamine hydrochloride (HN2) or triethylene melamine (TEM) intravenously.

TABLE 2
REMISSIONS OF AT LEAST THREE MONTHS' DURATION OBTAINED WITH R48

Disease	No. of cases treated	No. showing remissions of 3 mo. or longer
Hodgkin's disease	13	3
Reticulum cell sarcoma	9	0
Mycosis fungoides	3	0
Giant follicle lymphoma	8	1
Lymphosarcoma	7	5
Acute leukemia	4	0
Chronic lymphatic leukemia	29	15
Chronic myeloid leukemia	7	0
Multiple myeloma	8	1
Miscellaneous carcinoma	4	0
Total	92	25

The clinical usefulness of R48 is illustrated by the following case report (FIGURE 1).

F. G., aged 67, had a several-months' history of fatigue and had enlargement of lymph nodes. He showed pallor and marked enlargement of lymph nodes in the cervical, axillary, and inguinal areas. The spleen was enlarged to the level of the umbilicus. His hemoglobin was 11 gm. and his white cell count 30,000, of which 80 per cent were lymphocytes. The marrow was packed with lymphocytes. R48 therapy was started, using 400 mg. daily, and this dose was continued for about 6 weeks. The lymph node enlargement disappeared and the spleen became impalpable. The hemoglobin began to rise and within 3 months was 15 gm. Following an interval of 6 weeks without treatment, R48 was again started in a dosage of 100 mg. on alternate days; this was carried on for a further 9 months. Therapy was then stopped. Two months later the hemoglobin was observed to have fallen, and the white count to have risen to 15,000, with an increase of lymphocytes in the differential count. Treatment was started again, with subsequent improvement. During the ensuing four years this patient had intermittent courses of R48 that satisfactorily controlled his symptoms—enlarged lymph nodes, splenomegaly, and the blood picture. Early in 1956 his condition rapidly deteriorated and further treatment was of no avail.

The fact that we did not observe a significant number of remissions in Hodgkin's disease may have been due, as previously stated, to the selection of cases for treatment. In those cases that responded favorably, remissions were not maintained for more than several months.

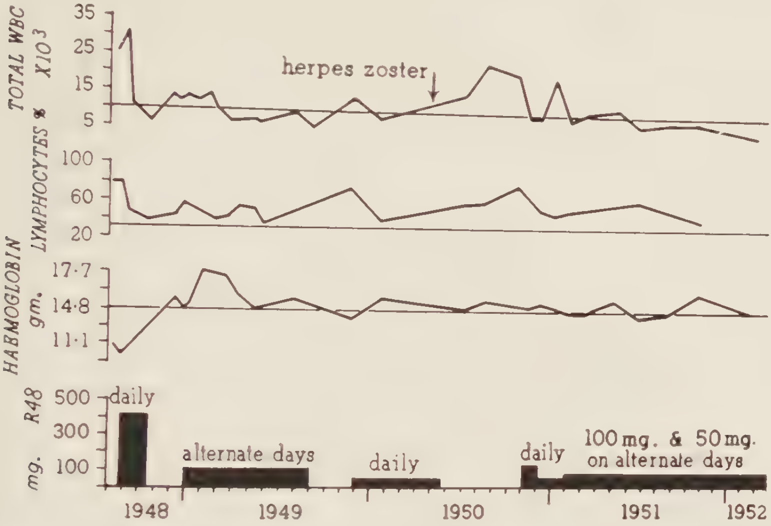

FIGURE 1. Patient F. G., 67-year-old man. Chronic lymphatic leukemia. Treatment with R48.

In reticulum cell sarcoma and in mycosis fungoides, the administration of R48 was not followed by the relief of symptoms sometimes observed with other alkylating agents. We were surprised that R48 did not give satisfactory remissions in giant follicle lymphoma, in contrast to the relatively good response in patients with the closely allied lymphosarcoma and chronic lymphatic leukemia.

That no response was observed in patients with chronic myeloid leukemia again may have been due to the fact that the patients treated were in the later stages of their disease.

In our experience, it is a fair generalization to say that a lesion that has not responded to radiation therapy seldom responds to chemotherapy. However, one is often faced with the problem of having to do something for a patient when radiation has failed; the following report illustrates a case in point.

S. F., aged 36, had a plasmacytoma of the trachea that failed to respond to radiotherapy. Tracheotomy and repeated excision of tissue were necessary to maintain life. R48 was given in a dosage of 400 mg. daily for a month, during which time the tumor vanished and it became possible to close the tracheotomy. During the same period a soft tissue mass in the vault of the skull melted away. This quite dramatic response lasted for almost a year. When recurrence did take place, the tracheal and skull tumors proved quite resistant to R48. Radiation was effective on the skull lesion, but not on the trachea.

The trial of another, closely related alkylating agent was carried out at the Royal Infirmary, Edinburgh. This compound, known as R151 (β-naphthyl-di-2-chloropropylamine),[6] was used orally in half the dose of R48, in 58 cases. It behaves like R48 and, as shown in TABLE 3, results comparable to those with R48 were observed.

TABLE 3
REMISSIONS OF AT LEAST THREE MONTHS' DURATION OBTAINED WITH R151

Disease	No. of cases treated	No. showing remissions of 3 mo. or longer
Hodgkin's disease	7	3
Reticulum cell sarcoma	7	0
Giant follicle lymphoma	6	0
Lymphosarcoma	6	2
Acute leukemia	3	0
Chronic lymphatic leukemia	11	5
Chronic myeloid leukemia	9	2
Multiple myeloma	4	0
Megakaryocytic leukemia	1	1
Polycythemia vera	1	1
Miscellaneous carcinoma	3	0
Total	58	14

R151 has also been used in combination with urethan in the treatment of multiple myeloma.[7]

Summary

(1) *NN*-di-2′-chloroethyl-2′-naphthylamine (R48, now known as CB 1048) has been used clinically in 92 patients, of whom all except 4 were suffering from malignant lymphoma, leukemia, or myeloma.

(2) The agent was given orally in doses ranging from 400 mg. daily as an initial dose to 25 mg. daily as a maintenance dose.

(3) Anorexia, nausea, vomiting, diarrhea, dysuria with frequency of urination, skin rash, and marrow depression have been the toxic effects observed.

(4) The agent is relatively slow in action.

(5) The agent was found to be useful in the management of approximately half of the 36 cases of chronic lymphatic leukemia and lymphosarcoma treated. Its use in other conditions was disappointing.

(6) A closely related alkylating agent β-naphthyl-di-2-chloropropylamine (R151) was used in another 58 patients, with comparable results. The dosage was one half that of R48.

Acknowledgment

The authors thank A. Haddow and the Boots Pure Drug Company for the supplies of material made available for testing. Appreciation is also expressed to members of the various services of the Royal Infirmary, Edinburgh, and the Toronto General Hospital who cooperated in this study.

References

1. Ross, W. C. J. 1949. J. Chem. Soc. :183.
2. Haddow, A., G. A. R. Kon & W. C. J. Ross. 1948. Nature. **162**: 824.
3. Matthews, W. B. 1950. Lancet. **1**: 896.
4. Gardikas, C. & J. F. Wilkinson. 1951. Lancet. **1**: 137.
5. Iversen, K. & E. Meulengracht. 1951. Brit. Med. J. **2**: 510.
6. Everett, J. L. & W. C. J. Ross. 1949. J. Chem. Soc.: 1972.
7. Innes, J. & W. D. Rider. 1955. Blood. **10**: 252.

STUDIES OF PHENYLALANINE NITROGEN MUSTARD (CB 3025) IN METASTATIC MALIGNANT MELANOMA OF MAN

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Phenylalanine is oxidized to tyrosine, to dihydroxyphenylalanine and, through subsequent steps of oxidation and polymerization, to melanin. Melanin pigment, however, is only an incidental concomitant of melanoma. Nonpigmented melanomatous tumors and portions of tumors exist. Nevertheless, we hoped that the importance of phenylalanine and tyrosine in the intracellular biochemistry of malignant melanoma might afford us an opportunity to introduce a chemical analogue of phenylalanine with some enhanced selectivity for the tumor.

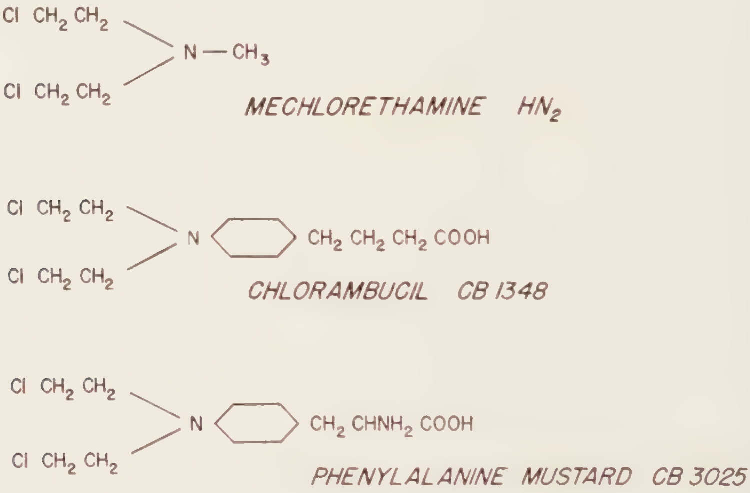

FIGURE 1

Bergel and his associates synthesized the *levo* phenylalanine nitrogen mustard, CB 3025 (FIGURE 1). Its relationship to the phenylbutyric mustard, CB 1348, is shown. The bis(β-chloroethyl)amino group constitutes not only the active mustard configuration, but a molecular modification, albeit a large one, which might enable the drug to be considered as an antimetabolite of phenylalanine. We hoped that the compound might become incorporated into the intracellular pathways of melanin synthesis and thus exert major inhibitory effect on melanoma cells.

The compound was shown to be highly active in Walker carcinoma 256 by Bergel and Stock.[1] After our clinical studies began, Luck[2] reported inhibition of Harding-Passey melanoma in a small number of mice by phenylalanine nitrogen mustard.

Sixteen of 18 patients with malignant melanoma admitted to the Medicine A Service of the Roswell Park Memorial Institute during a 14-month period in 1955 and 1956 were included in the study.* Two patients with minimal evidence of recurrent disease were not subjected to drug administration because they were able to carry on normal activities at home. In all our patients metastatic malignant melanoma was proved by biopsy. Only one patient had had other alkylating compounds.

Phenylalanine nitrogen mustard was administered orally in all cases in 2-mg. tablets generously provided by A. Haddow. The drug was ordinarily administered in courses of 50 to 100 mg. each. Eight patients received 3 or more courses; 1 patient received 7. The daily dose and the median dose for the first course are shown in TABLE 1. The total drug administered throughout the study ranged from 30 to 472 mg. in a treatment period (delineated by the first and last days on which the drug was administered) of 3 to 397 days. The median total dose for all patients was 207 mg. in 41 days.

TABLE 1

PHENYLALANINE NITROGEN MUSTARD: FIRST COURSE OF ADMINISTRATION

	Daily dose		Total dose		Days
	mg.	mg./kg.	mg.	mg./kg.	
Range	10–20	0.089–0.37	30–120	0.55–2.22	3–17
Median	—	—	100	1.41	5

Nine of 16 patients vomited during the first course when, as was our usual practice, the drug was administered in one dose, fasting. In subsequent courses, these patients vomited less when tablets were taken individually with food throughout the day. On occasion, anorexia persisted for several days. In 4 patients no gastrointestinal abnormalities were seen throughout repeated courses.

The most important toxic manifestations encountered (TABLE 2) were leukopenia (<4000 per cu. mm.) and thrombocytopenia ($<100{,}000$ per cu. mm., or 50 per cent of the patient's initial level if it was $<100{,}000$). In the 6 patients who developed leukopenia the minimum white cell count was observed from 6 to 41 days after drug administration, with a median of 17 days. In 14 of the 16 patients there was appreciable reduction in leukocyte count, although not to the level defined for leukopenia. Platelet depressions, as defined, occurred in 6 patients, and minimum counts were seen from days 13 to 31. The median total dose for the first course in those patients who experienced hematopoietic depression was greater (1.54 mg./kg.) than in the 9 patients who experienced neither leukopenia nor thrombocytopenia (1.22 mg./kg.). No serious infections and no major bleeding occurred during the cytopenic periods. No fatal drug intoxication was encountered. Return to pretreatment levels of leukocytes and platelets occurred rapidly. A subsequent course of drug was not administered until cytopenia from the effects of the preceding dose had abated. The median interval between the first and second course in those 13 patients who were treated a second time was 3 weeks. No cumulative toxicity was apparent, and subsequent hematopoietic depressions were not more severe than preceding ones.

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* The period of observation was from Oct. 14, 1955, to Feb. 1, 1957.

TABLE 2

CYTOPENIA FROM PHENYLALANINE NITROGEN MUSTARD

	Total	Leukocytes <4000		Platelets <100,000	
		First course	All courses	First course	All courses
Patients	16	6	9	6	9
Courses	42	—	18	—	15

Fourteen of the 16 patients observed showed no evidence of tumor regression and no clinical improvement. One patient was removed from the series after only 30 mg. of the drug, and radical surgical removal of a pelvic mass was performed. No evidence of drug effect on the tumor was seen. In the other 13 failures, 12 developed new metastases while receiving the drug, and all had progressive increase in tumor size of the metastases being measured. In none of the 16 patients was change in pigmentation of the metastases observed.

In the remaining 2 of the 16 patients, objective evidence of limited benefit was seen. In one man, J. McK., massive hepatic metastases from melanoma of the chorioid had occurred. On admission, his serum alkaline phosphatase activity and serum bilirubin concentration were moderately elevated. During therapy with five courses of the drug, decline in these abnormalities toward normal occurred. Following a sixth course of the drug, administered after the termination of the study period under consideration, there was continuing decrease of bilirubin concentration to normal, and of alkaline phosphatase activity, which was then only slightly elevated. After having constantly gained weight attributable to ascitic fluid, pleural effusion, and peripheral edema for 150 days on treatment he spontaneously diuresed 9 kg. without change in albumin concentration. No decrease in hepatic size was noted. No new lesions appeared. Throughout the treatment period he sustained neither leukopenia nor thrombocytopenia.

The second patient in whom objective improvement has been noted was a 64-year-old woman, E. R. Recurrent melanoma was present in the abdominal wall, in an axillary node, and an enlarged liver was present. Sixty-four days after 100 mg. of phenylalanine nitrogen mustard in her first course (1.85 mg./kg. in 10 days), decrease in the size of the tumor nodules was evident in serial measurements and photographs. Progressive decrease in all lesions continued and persisted. The patient's liver decreased in size. Ten months after the initiation of drug administration, increases in the size of the lesions began again, and a metastasis was observed for the first time on her left arm. She again received courses of the drug which produced depression of leukocytes and platelets.

It should be noted that this patient, who attained the most impressive objective change in her melanoma, also sustained the greatest depression of leukocyte count of all the patients after her first course (from 8700 to 850 per cu. mm.). Her platelet count also dropped from 206,000 to 35,000. It is inviting to speculate that the association of the most certain objective tumor regression and the greatest hematopoietic depression may not be coincidental.

In addition to the melanomas presented, we have studied a few patients with other disseminated neoplasms. Two patients with breast cancer and one with cancer of the cervix showed no tumor response. Minor decreases in the size of the metastases were seen in one patient with metastatic bronchogenic carcinoma. Suboptimal control of chronic lymphocytic leukemia was attained in one man. In another man, nearly complete necrosis of all metastases from embryonal carcinoma of the testis was found at autopsy shortly after drug administration was completed. The doses were similar to those employed in patients with melanoma.

In summary, we have noted some improvement in 2 of 16 patients with metastatic malignant melanoma during the period when phenylalanine nitrogen mustard was being administered. Whether this constitutes therapeutic response or fortuitous association is not clear. The greatest tumor effect was seen in the patient who demonstrated the greatest hematopoietic depression, which suggests that the two phenomena are related. The drug has been administered at dose levels that caused recognizable decrease in leukocytes in 14 of 16 patients. The therapeutic activity of oral phenylalanine nitrogen mustard in malignant melanoma in man is, at best, of low order.

Since these observations, we have given 12 courses of intravenous therapy in 6 patients with metastatic malignant melanoma. A usual course was a total of 100 mg. administered in 4 days. No therapeutic benefit was seen. Toxic reactions were entirely similar to those noted after oral doses.

References

1. Bergel, F. & J. A. Stock. 1953. 31st Ann. Rept. Brit. Empire Cancer Campaign. : 6.
2. Luck, J. M. 1956. Science. **123**: 984.

PRELIMINARY CLINICAL TRIAL OF *p*-DI-2-CHLOROETHYLAMINO-L-PHENYLALANINE (CB 3025, MELPHALAN) AND OF DI-2-CHLOROETHYL METHANESULFONATE (CB 1506)

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*p-Di-2-Chloroethylamino-*L*-Phenylalanine (CB 3025, Melphalan)*

Results of preliminary clinical trial. This substance has been given to 37 patients suffering from advanced malignant disease. The oral route was used in 30 cases and the intravenous route in 7. Orally, the drug was well tolerated in doses less than 20 mg.; higher doses led to nausea or vomiting. Neutropenia and thrombocytopenia followed 2 to 3 weeks' treatment with doses of 10 mg. daily, and 8 to 10 weeks' treatment with doses of 4 to 6 mg. daily. Recovery was prompt after discontinuing treatment. Intravenously, the drug induced nausea and vomiting in 2 patients. The veins were not damaged. The doses used were 25 mg. in 6 cases, 50 mg. in 1 case. Two to 4 injections were given on successive days and were repeated from 1 to 6 weeks later. As many as 4 courses have been given in 11 weeks. All patients suffered some degree of neutropenia and 3 experienced mild thrombocytopenia; all recovered quickly.

Results: intravenous administration. There were 2 cases of teratoma testis in young adults, 2 of reticulosarcoma, and 1 each of Hodgkin's disease, acute leukemia, and malignant melanoma. One patient with teratoma testis derived considerable benefit. He received 250 mg. of CB 3025 in 3 months. Pain was relieved, a large upper abdominal mass regressed considerably, he gained 9 lb. in weight, and his hemoglobin level increased by 4.7 gm. per 100 ml. in 3 months. Transient regressions were observed in the 2 cases of reticulosarcoma, and there was short-lived symptomatic relief in the patients with melanoma and Hodgkin's disease.

Results: oral administration. Thirteen patients suffering from Hodgkin's disease were treated, as well as 6 cases of reticulosarcoma, 3 of malignant melanoma, 2 of bronchial carcinoma, 2 of gastric carcinoma, and 1 each of carcinoma of the breast, ovary, pancreas, and prostate.

In 7 cases of Hodgkin's disease there were transient effects on fever, sweats, pruritus, and on the size of the involved lymph nodes, but 4 patients subsequently responded better to chlorambucil, and one also to triethylene melamine (TEM); one patient who did not respond at all to melphalan, although neutropenia and thrombocytopenia occurred, later responded to mustine. Melphalan is inferior to chlorambucil and to mustine in the treatment of Hodgkin's disease.

In breast carcinoma transient slight regression of skin nodules but not of malignant ascites took place. In prostatic carcinoma absorption of pleural effusion with relief of dyspnea that lasted three months occurred; there was no response to a second course of treatment. The remaining patients did not respond to Melphalan.

2-Chloroethyl Methanesulfonate (CB 1506)

Twenty-eight patients have been treated: 13 cases of bronchial carcinoma, 5 of reticulosarcoma, 3 of Hodgkin's disease, 2 of malignant melanoma, and 1 each of mammary, prostatic, and large bowel carcinoma, salivary adenocarcinoma, and fibrosarcoma of the ovary.

CB 1506 is a colorless oil soluble in 50 times its volume of water. It was given orally to 26 patients in solution in 3 cases and in plain or enteric-coated capsules in 23 cases. The daily dose ranged from 0.25 gm. to 1.25 gm., and the maximum total dose was 63.5 gm. Three patients, including one who had already taken the drug by mouth, received CB 1506 intravenously in doses of 0.25 gm. dissolved in 20 ml. saline. The 3 patients who took the solution by mouth all experienced nausea, and 2 vomited. The capsules, both plain and enteric-coated, were slightly better tolerated, but 17 patients complained of nausea or vomiting. This was not clearly related to dose, for a few patients were nauseated when taking only 0.25 gm. daily, while others took 1 gm. daily for many weeks without complaint. Some, but not all, intravenous injections of CB 1506 induced slight nausea.

Results. In 2 cases of bronchial carcinoma slight regressions of involved nodes and of a skin nodule were recorded. Similar effects were noted in the patient with mammary carcinoma with multiple skin deposits, in the patient with ovarian fibrosarcoma, and in the patient with salivary adenocarcinoma. In 2 cases of Hodgkin's disease, transient effects on fever and on involved nodes were observed. In none of these cases were the effects of any clinical significance.

CLINICAL EXPERIENCES WITH SARCOLYSIN IN NEOPLASTIC DISEASES

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Experimental investigation of sarcolysin [DL-*p*-di(2-chloroethyl)aminophenylalanine hydrochloride], which was synthesized in 1953,* indicated that the compound produces a complete regression of three strains of transplantable rat and mouse sarcoma and an inhibition of growth of other sarcomas and carcinomas.

The clinical trial of sarcolysin was begun in June 1954. At present we have clinical data on the results of sarcolysin treatment of some 200 patients, 150 of whom underwent treatment in the research hospital of our institute; the rest received treatment in other institutions under our supervision.

From the very beginning, the clinical use of sarcolysin has shown that there is a definite difference in the spectrum of its action from that of nitrogen mustards. In contrast to HN2, it was found that sarcolysin had no influence on Hodgkin's disease and lymphosarcoma, whereas it exerted a positive effect on certain solid tumors.

Clinical experience has indicated that the following tumors are most sensitive to sarcolysin: seminoma of the testis (especially its metastases in lymph nodes), reticulum cell sarcoma, Ewing's tumor of bone, and thymoma. A single course of sarcolysin treatment produced a complete regression of tumor in a number of patients.

Less sensitive to sarcolysin but definitely responsive are: mixed tumors of the testis (seminoma with elements of malignant teratoma), lymphangio- and angioendothelioma, multiple myeloma, and cholangiocell carcinoma of the liver. A number of the above-mentioned tumors underwent partial regression as a result of a single course of sarcolysin treatment.

It was found that the following tumors are generally insensitive to the drug: chorioepithelioma and teratoma of the testis, malignant melanoma, fibro-, chondro-, and polymorph cell sarcoma, primary osteogenic sarcoma, hepatocellular carcinoma of the liver, hypernephroma, gastric and pancreatic carcinoma, and primary bronchogenic and mammary carcinoma.

Table 1 gives data on tumors sensitive and relatively sensitive to sarcolysin.

Our data show that various tumors of the same organ having different histological structures may differ greatly in their response to sarcolysin. For example, seminomas are very responsive, mixed tumors of the testis less responsive, and chorioepitheliomas and teratomas not responsive at all.

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* An independent investigation was carried out at the same time by F. Bergel, J. A. Stock, and A. Haddow.

TABLE 1

Tumor	No. of patients	Immediate positive result		Negative result
		Complete regression of tumors	Considerable reduction of tumor size	
Seminoma and its metastases	23	11	8	4
Reticulum cell sarcoma	16	2	6	8
Ewing's tumor	13	4	6	3
Thymoma	1	1	—	—
Mixed tumors of the testis	9	—	5	4
Angioendothelioma	10	—	4	6
Primary carcinoma of liver	10	—	3	7
Multiple myeloma	6	—	3	3
Total	88	18	35	35

These differences may even serve to a certain extent for a differential diagnosis based on the results of treatment.

It is also necessary to stress the very important fact that the sensitivity of primary tumors to sarcolysin may differ from that of their metastases. As a rule, the metastases are more responsive than primary tumors. For example, the primary seminoma is less responsive than its lymphogenic metastases. In view of this fact we removed the primary tumor even after the complete regression of its metastases. A second example: the primary osteogenic sarcoma and the bronchogenic carcinoma are insensitive to the drug, but initial data indicate that lung metastases of osteogenic sarcoma may disappear.* We have also observed a regression of secondary local nodes of osteogenic sarcoma after the removal of the primary tumor. Initial positive results were observed also in cases of the metastases of bronchogenic carcinoma in lymph nodes* and of the mammary carcinoma in the skull bones.†

Data on the difference in the response of different types of metastases were also obtained. For example, the lung metastases of seminoma are definitely less sensitive than lymphogenic metastases of the same tumor.

After the primary cure of seminoma, reticulum cell sarcoma, Ewing's tumor, and thymoma, a relapse was observed in a number of patients, but in the majority of cases these tumors responded to repeated treatment. To date no return of the disease after a single course of treatment has been observed in 3 patients with seminoma metastases (for 1 to 2½ years), in

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* Data submitted by I. S. Ginsburg, Oncological Department, Postgraduate Medical School, Baku.

† Treatment supervised by us in the Oncological Dispensary, Minsk.

2 patients with Ewing's tumor (for 6 months to 1½ years), and in 1 patient with reticulum cell sarcoma (for 1 year).

TABLE 2 gives data on patients apparently cured at the present time. The table includes the cases mentioned above, as well as cases with successful treatment of relapse.

TABLE 2

Tumor	No. of patients	Period of observation
Metastases of seminoma (primary tumor removed)	7	From 6 months to 2½ years
Ewing's tumor	3	Up to 1½ years
Reticulum cell sarcoma	1	1 year
Multiple myeloma	1	1 year

In other cases of responsive tumors the effect of treatment was temporary. It was followed by relapse and progress of the disease.

The results of treatment are determined, not only by the sensitivity of tumors to sarcolysin, but also by the method of drug administration, which is a very essential factor.

We administer sarcolysin orally and, in rare cases, intravenously. Our experimental data served as the basis for the application of large doses of sarcolysin, especially at the beginning of treatment, with long intervals (of 1 week). For the majority of patients the initial one or two doses of sarcolysin consist of 50 mg. once a week. Subsequent doses are diminished to 40 mg. (in some cases to 30 mg.), with the same intervals. The determining factor in dosage is the reaction of the hematopoietic system; that is, the decline in the number of leukocytes.

A 40-mg. initial dose is administered to patients weighing less than 60 kg., to patients who have undergone intensive X-ray therapy, and to patients with weakened hematopoiesis. Subsequent dosage, if it is found necessary, is diminished to 30 mg.

The course of treatment usually consists of from 5 to 7 doses of sarcolysin. The total dose is from 150 to 250 mg. The treatment is discontinued when the number of leukocytes falls to 3500 to 3000 per cu. mm.

Clinical experience shows that treatment should not be limited to one course. If the tumor undergoes a significant regression but does not completely disappear, an additional course of treatment should be conducted after an interval of 1 to 1½ months; that is, after the regeneration of white blood cells. The second course of treatment serves to achieve complete regression of the tumor and, if this course proves insufficient, then a third course may be administered.

If the first course of treatment brings about complete regression of the tumor it is advisable to carry out a minimum of one prophylactic course without waiting for a relapse. Standard single doses are administered in the

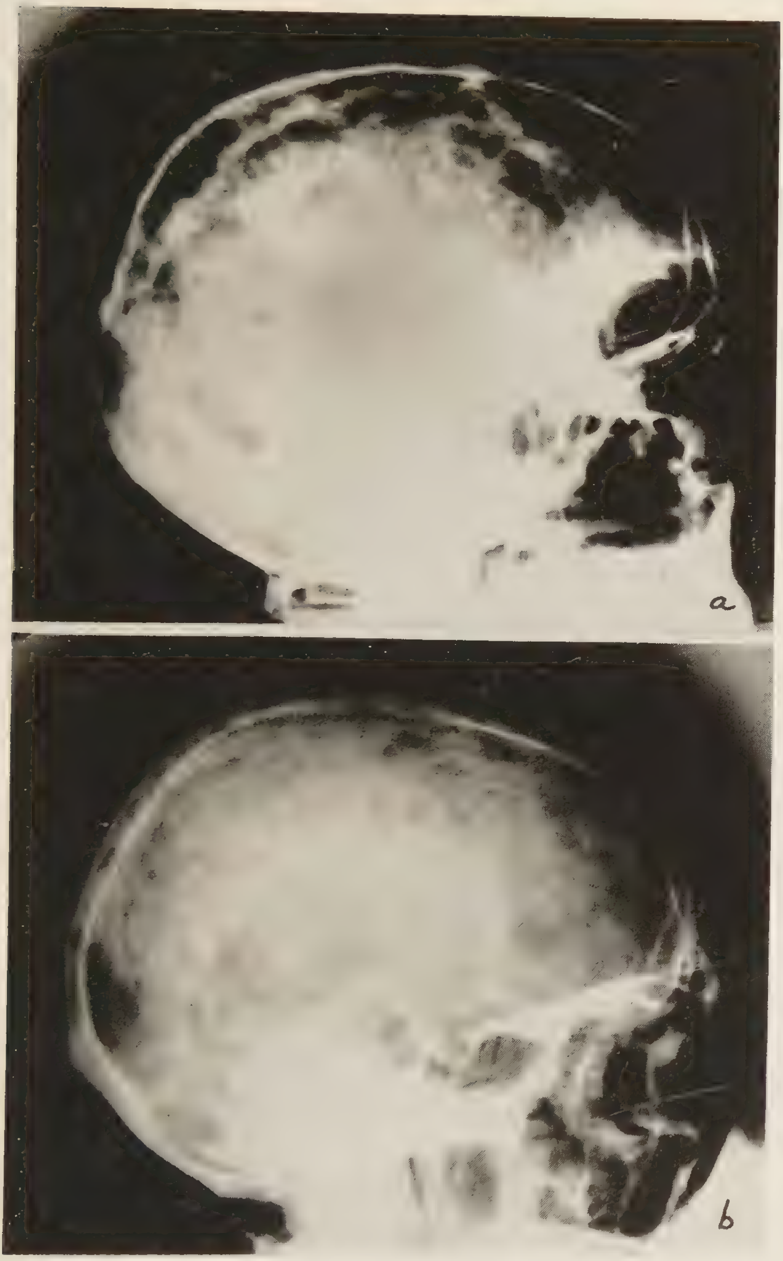

FIGURE 1. Patient with multiple myeloma: (*a*) before treatment; (*b*) after treatment vith sarcolysin.

prophylactic course, but the number of doses is reduced (3 to 5 doses instead of 5 to 7). In case of a relapse, a repeated course should be carried out immediately upon detection of its first symptoms.

A number of patients with sarcoma of the extremities have received intra-arterial injections of sarcolysin. This method of administration proves to be effective in cases of tumors responsive to the drug.

To illustrate the possibilities of persistent treatment we may cite the case of a patient with myelomatosis. Administration of sarcolysin was begun when the patient was in a very critical state, with multiple involvement of the skull, ribs, and pelvic bones, after the failure of radioactive phosphorus treatment. The patient's hemoglobin level was 27 per cent (Sahli), and there was 20 *pro mille* protein content in the urine.

Three courses of sarcolysin treatment were carried out in succession with intervals of from 1 to 2 months.* The first course was conducted with doses of 30 to 40 mg. (total dose, 210 mg.). The single dose in the second and third course was enlarged to 50 mg. The total dose for the last two courses was from 250 to 270 mg. Because leukopenia occurred, repeated transfusions of blood and of suspensions of leukocytes and thrombocytes were carried out during and after treatment.

At the present time—that is, one year after the beginning of treatment and 6 months after the end of the third course—the patient has apparently recovered. He has gained 20 kg. in weight and is able to carry on intensive work as the director of a large plant. In the majority of bone defects a regeneration of bone tissue has occurred (FIGURE 1).

To sum up, sarcolysin seems to be the first drug of the chloroethylamine group that produces a definite curative effect on certain solid malignant tumors and is ineffective in the treatment of Hodgkin's disease. It is regrettable that the curative action is limited to rare forms of tumors.

There is reason to believe that new drugs, different in their spectrum of action, may be obtained by means of the substitution of phenylalanine in sarcolysin by other carriers of the chloroethylamine group. Research in this field is now under way.

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* The patient received treatment under our supervision in the Sverdlov Hospital, Leningrad.

INITIAL CLINICAL TRIAL OF THE NITROGEN MUSTARD ANALOGUES OF CHLOROQUINE AND QUINACRINE: A PRELIMINARY REPORT*

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INTRODUCTION

Extensive studies of the fate and tissue distribution of the antimalarial drugs chloroquine and quinacrine, conducted during World War II and subsequently, have revealed that these compounds concentrate selectively in certain parenchymatous organs.[1-3] Within the cell, these drugs localize and accumulate in the nucleus; this has been thought to be due to the fact that they have a selective affinity for nucleic acid and, in particular, for deoxyribonucleic acid.[4] *In vitro* and *in vivo*, both agents form a relatively stable complex with nucleic acids.

During World War II a great many 9-amino acridines related to quinacrine, and many 4-amino quinolines related to chloroquine, were synthesized and subjected to detailed pharmacological study, including studies of tissue distribution in experimental animals. Thus, for compounds of these two types there exists a large body of information about the relation of chemical structure to the property of selective concentration in certain tissues.[3] It has been shown that minor modification of chemical structure may profoundly change the pattern of tissue distribution of these compounds and their affinity for nucleic acid.[5]

Chloroquine and quinacrine each possess a side chain that terminates in a tertiary diethylamino group. The similarity of this structure to the alkylating agent nitrogen mustard suggested that the substitution of a chlorine atom for a hydrogen atom on each of the terminal diethyl groups might produce an alkylating agent with properties similar to those of nitrogen mustard (FIGURE 1). If this were true, it would be of interest to determine whether or not these compounds might concentrate selectively in malignant tumors arising from tissues in which the parent compound accumulated, carrying the alkylating activity of the terminal group into the malignant tumor in high concentration.

The synthesis of the nitrogen mustard analogues of chloroquine, quinacrine, and pamaquine has been described,[6] and the new compounds have been shown to possess the chemical properties that characterize alkylating agents. In various biological systems they produce effects similar to those produced by nitrogen mustard and other alkylating agents; for example, filament formation in *Escherichia coli*, lysogeny in phage-infected bacteria, and mitotic inhibition.[7]

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† Clinical Fellow, American Cancer Society.

7-Chloro-4-[4-bis(β-chloroethyl)amino-1-methylbutylamino]quinoline ("chloroquine mustard") and 2-methoxy-6-chloro-9-[4-bis(β-chloroethyl)-amino-1-methylbutylamino]acridine ("quinacrine mustard") have been found to produce marked inhibition of certain ascites tumors in mice,[8] and significant inhibition of AK-9 leukemia in mice and IRC-741 leukemia in rats.[9] Because of this evidence of chemotherapeutic activity against malignant tumors in experimental animals, chloroquine mustard and quinacrine mustard have been subjected to pharmacological studies in mice, rats, dogs, and monkeys,[10] and we have conducted studies of human pharmacology and preliminary therapeutic trial in patients with various types of malignant disease. It is our purpose to present a preliminary report of the results of studies of the effect of these agents in man.

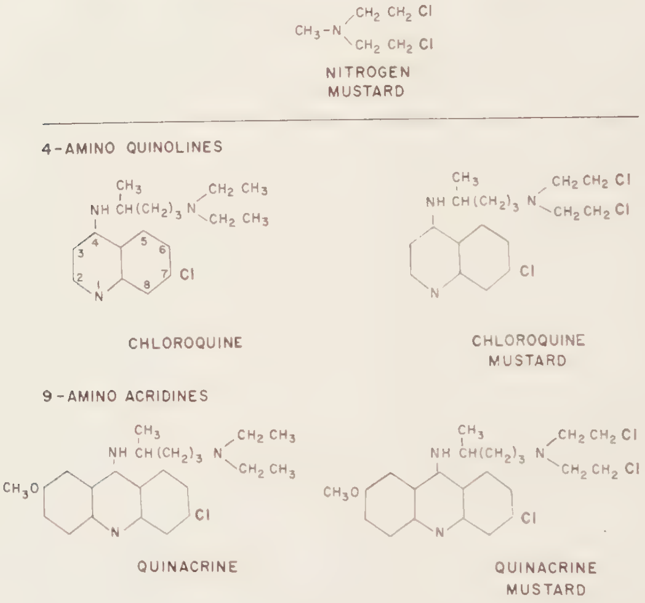

FIGURE 1

CHLOROQUINE MUSTARD

Method of Study

The drug. Only the HCl salt of chloroquine mustard was used in these studies. Initially the drug was synthesized by Price and Sen in the Harrison Laboratories of the University of Pennsylvania, and was supplied in bulk. In this form it was extremely hygroscopic. It was stored in the cold in a vacuum desiccator, and was removed only to weigh out the appropriate dose. The drug was dissolved in normal saline for parenteral administration. A fresh solution was prepared each day and the drug was administered promptly after the solution was made. Subsequently the drug was synthesized and supplied as a dry powder in single-dose ampoules by the Sterling-Winthrop Research Laboratories, Rensselaer, N. Y.

The activity of each lot of drug was determined by Creech and his associates in mice with ascites tumors by the methods described elsewhere in this publication[8] before the material was used in man.

Selection of patients. Only patients with advanced incurable malignant disease, not amenable to palliative therapy with surgery, radiation therapy, or other specific chemical agents, were included in these studies. Only patients with lesions demonstrable by objective methods were studied.

Procedure. For all patients a detailed history was taken, a thorough physical examination was done, and several days of clinical observation were instituted before treatment was initiated. Superficial lesions were measured in two diameters by calipers, and X-ray studies were done, as indicated, to document the status of lesions demonstrable by this method. All patients had complete blood counts, bone marrow examinations, and selected studies of hepatic and renal function, and they were examined twice daily during the period of treatment. The blood count was repeated three times a week, and superficial lesions were measured three times weekly. Other laboratory and X-ray studies and bone marrow examinations were repeated, as indicated, during treatment.

In each patient the initial dose of the drug was approximately one tenth the maximum tolerated dose in the most susceptible species of experimental animal. This dose was increased gradually during the period of treatment until evidence of toxicity was encountered or evidence suggestive of therapeutic activity was obtained.

Pharmacological Effects

Intravenous administration. Chloroquine mustard hydrochloride was administered intravenously to 4 patients with far-advanced malignant disease (TABLE 1). Single initial doses of 0.1 to 0.2 mg./kg. produced no detectable effect. Two patients received an initial dose of 0.1 mg./kg., and a dose of 0.3 mg./kg. on the following day, without evidence of any untoward effect. On the fourth day, the dose was inadvertently increased to 0.5 mg./kg. Forty-five minutes later both patients developed grand mal convulsions, which lasted for 12 minutes. When the convulsions subsided, each patient xhibited continuous aimless motor activity, which subsided gradually over he next 24 hours. During this period they were disoriented as to time, place, and person and showed clear evidence of hallucinations and delusions. At times the hallucinations had a color component and were described as vivid reds and greens. Forty-eight hours after the convulsion, the hallucinations ceased, and both patients were completely oriented. Each retained remarkably clear memory of the entire episode from approximately 15 minutes after the end of the convulsion.

TABLE 1

TOXICITY OF CHLOROQUINE MUSTARD IN MAN

Patient	No. of days of treatment	Maximum single dose mg./kg.	Total dose mg./kg.	Toxic reactions
		Intravenous administration		
H. C.	5	0.1	0.5	Paresthesias, psychosis
C. K.	3	0.3	0.6	Psychosis
M. G.	3	0.5	0.9	Convulsion, psychosis
R. B.	3	0.5	0.9	Psychosis
		Intramuscular administration		
D. K.	5	0.2	1.0	
C. K.	16	0.2	1.2	
H. W.	22	0.2	1.7	
M. R.	13	0.2	2.7	Local reaction
J. C.	27	0.2	2.7	
E. B.	18	0.2	3.8	Psychosis
C. L. A.	40	0.2	4.0	
M. P.	36	0.24	4.2	
M. G.	43	0.2	5.1	
E. G.	6	0.3	1.4	Local reaction
M. C.	12	0.3	1.8	
A. T.	23	0.3	2.1	
D. E.	29	0.3	4.4	Local reaction
G. C.	24	0.3	6.4	Thrombocytopenia
K. A.	58	0.3	6.9	
S. N.	42	0.3	10.3	
K. H.	27	0.4	10.3	Leukopenia
E. B.	39	0.4	10.2	Nausea and vomiting
M. U.	33	0.43	4.1	Convulsion
J. L.	73	0.5	11.7	Convulsion
S. R.	69	0.6	11.1	Local reaction
G. T.	49	0.8	15.8	Convulsion

A third patient received an initial dose of 0.1 mg./kg. followed by a dose of 0.2 and one of 0.3 mg./kg. on successive days. Approximately 30 minutes after the third dose, the patient became confused and disoriented, and developed hallucinations and continuous aimless motor activity. This subsided over a period of 47 hours, and the patient retained a fairly clear memory of the event.

A fourth patient was given a single daily dose of 0.1 mg./kg. for 5 successive days. After the fourth dose he complained of diffuse painful paresthesias that lasted approximately 3 hours. Thirty minutes after the fifth dose, painful paresthesias developed and the patient became confused and disoriented, and developed hallucinations of an unpleasant character. Eight hours later all symptoms had subsided and the patient retained a clear memory of the entire period.

No untoward effects other than those related to the central nervous system were observed in these patients.

Intramuscular administration. Chloroquine mustard has been administered intramuscularly to 20 patients with far-advanced malignant disease (TABLE 1) in doses ranging from 0.1 to 0.8 mg./kg. a day. The duration of treatment has varied from 5 to 73 days.

In 9 patients the maximum single dose was 0.2 mg./kg. It was necessary to discontinue treatment in one patient because of the occurrence of toxic psychosis. This patient received a daily dose of 0.1 mg./kg. for 10 days, followed by 0.2 mg./kg. every other day for 30 days. The psychosis, which was characterized by confusion, disorientation, and hallucinations, cleared in 24 hours after treatment was stopped. One patient developed areas of tender induration and inflammation at the site of each injection.

In 7 patients the maximum single dose was 0.3 mg./kg. All but 1 of these patients were children. One patient developed moderately severe thrombocytopenia, but this was accompanied by rapid progression of his disease, so it is not possible to be sure that this was due to the drug. One patient developed such severe local reactions that it became necessary to terminate treatment; a second patient developed less severe local reactions to the drug.

In 5 patients the dose was increased to 0.4 mg./kg. or more before limiting toxic reactions were encountered. In one patient the total white blood cell count fell to 1400/cu. mm. after 27 days of therapy. The white blood cell count returned to normal levels 10 days after treatment was discontinued. Throughout the period of study this patient was treated with phenobarbital in an attempt to prevent central nervous system toxicity.

One patient developed protracted nausea and vomiting when the daily dose was increased to 0.4 mg./kg. after 36 days of therapy; this subsided promptly when the drug was withheld for a few days. This patient later received a daily dose of 0.2 mg./kg. for 18 days without recurrence of the symptoms.

Three patients developed severe convulsions, accompanied by respiratory irregularities, 1 to 2 hours after injection of the drug. In 1 of these patients, the daily dose was increased from 0.1 to 0.5 mg./kg. over a period of 73 days. This patient had received a daily dose of 0.5 mg./kg. for 10 days without any untoward reaction. On the following day he developed a clonic convulsion lasting approximately 4 minutes. Forty-eight hours later, a single dose of 0.1 mg./kg. produced a mild convulsive seizure. In 1 patient, a 6-month-old infant, the daily dose was increased from 0.21 to 0.43 mg./kg. for 8 days without untoward effect. On the ninth day, this child developed a severe clonic-tonic convulsion lasting approximately 35 minutes, accompanied by marked respiratory embarrassment. He recovered completely and has been well for 8 months.

In a third patient, a 79-year-old male with lympho-leukosarcoma, the daily dose was increased from 0.1 to 0.8 mg./kg. over a period of 48 days. He had tolerated a daily dose of 0.8 mg. for 4 days. Forty-five minutes after the fifth dose of 0.8 mg./kg., he became confused and developed a severe clonic convulsion lasting approximately 8 minutes. This was followed by coma and shallow irregular respiration, which lasted for 2 hours.

Considering the occurrence of toxicity in relation to total dose: 7 patients received a total dose of 2 to 4 mg./kg. and none developed evidence of significant toxicity; 5 patients received a total dose of 4 to 8 mg./kg. and 3 of the 5 developed evidence of serious toxicity; 7 patients received a total dose of more than 8 mg./kg. and 5 of the 7 developed serious toxicity.

The limiting toxic reaction in all but 2 of the patients was the result of an effect on the central nervous system. In only 2 patients was there evidence of bone marrow depression, and these patients had received phenobarbital in an attempt to prevent central nervous system toxicity.

Preliminary Evidence of Chemotherapeutic Activity

The type of malignant disease that was present in the patients who were treated with chloroquine mustard and the change that occurred in the disease during the period of treatment are shown in TABLE 2. In 2 of the 5 patients with neuroblastoma who were treated with chloroquine mustard all evidence of the disease disappeared, and both patients have remained well for more than one year. In 3 patients with neuroblastoma there was no evidence of any therapeutic effect of the drug.

TABLE 2

RESULTS OF PRELIMINARY THERAPEUTIC TRIAL OF CHLOROQUINE MUSTARD IN MAN

Disease	Number of patients	Change in the disease during treatment		
		Marked improvement	Some improvement	No change
Neuroblastoma	5	2	0	3
Chronic leukemia				
Lymphatic	2	—	1	1
Myelogenous	2	1	—	1
Acute leukemia	3	1	1	1
Hodgkin's disease	2	—	1	1
Lymphosarcoma	2	—	—	2
Reticulum cell sarcoma	2	—	—	2
Bronchogenic Ca	3	—	—	3
Wilms's tumor	1	—	—	1
Astrocytoma	1	—	—	1
Rhabdomyosarcoma	1	—	—	1
Total	24	4	3	17

Case No. 1. K. A., a 5-month-old white female, developed progressive enlargement of the abdomen and anemia at the age of 2 months. Liver biopsy revealed extensive infiltration with neuroblastoma cells; the left kidney was depressed by a mass; and several bone marrow examinations showed typical rosettes of neuroblastoma.

The child was treated with X radiation to the abdomen for 3 weeks. There was a transient reduction in the size of the enlarged liver, followed by progressive enlargement over a 3-week period.

Four weeks after the last treatment with X ray, the child was given 0.1 mg. kg. of chloroquine mustard intravenously. During the next 2 months she received chloroquine mustard intramuscularly every other day. The dose was gradually increased from 0.1 mg. kg. to 0.3 mg. kg. without any untoward effect. At the end of 1 month of treatment the liver was palpably smaller, and the abdominal girth had decreased from 22 to 19 in. Her hemoglobin had increased 2 gm., and the child had regained her appetite and started to gain weight. When treatment was discontinued, the liver was approximately one third its previous size, and the child was continuing to improve. Treatment was discontinued because the supply of the drug was temporarily exhausted.

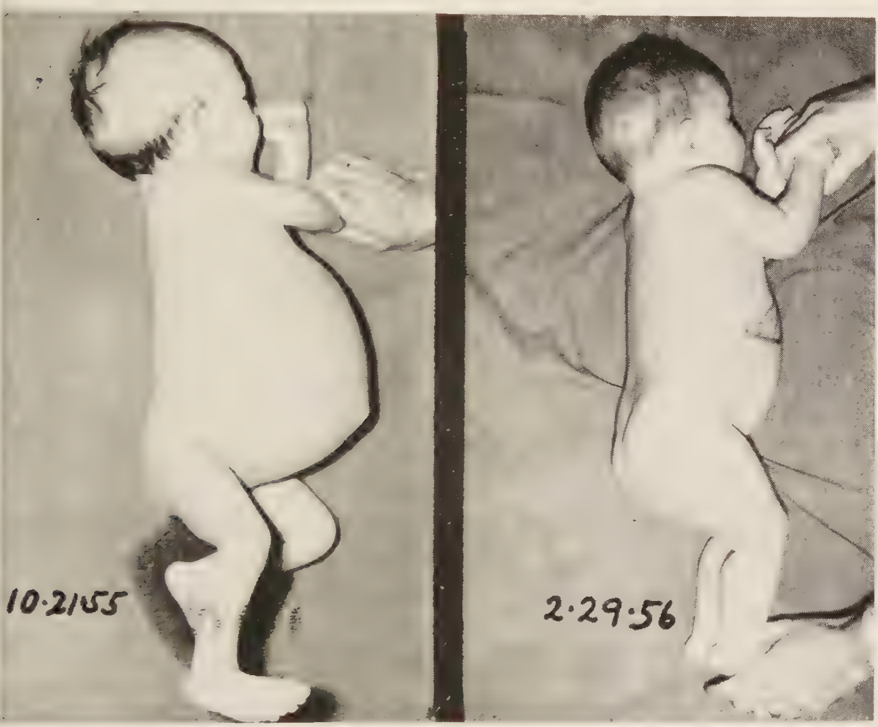

FIGURE 2. Patient K. A. Left: Before treatment with chloroquine mustard was started, showing protuberance of the abdomen due to massive enlargement of the liver. Right: two months after completion of treatment.

In FIGURE 2 is shown a photograph of this child before treatment was initiated and 2 months after treatment was terminated. The liver gradually returned to normal size, and the child has been free of all evidence of disease for a period of 20 months.

Case No. 2. M. U., a 3-month-old white male, was found to have a tumor in the skin of the perineum. This was biopsied and showed the characteristic picture of neuroblastoma. Repeated bone marrow examinations showed that the marrow was filled with the rosettes of neuroblastoma cells. An intravenous pyelogram revealed downward displacement of the left kidney, with evidence of a mass above. No lesions were seen in the bone survey.

The child was treated with chloroquine mustard intramuscularly over a period of 33 days. Treatment was initiated with a dose of 0.2 mg. kg. every other day, and the dose was gradually increased to 0.43 mg. kg. On the thirty-third day, the child had a severe convulsion, and treatment was discontinued.

At this time the hemoglobin had increased 1.5 gm.; two bone marrow examinations failed to reveal any neuroblastoma cells, and the mass above the kidney was smaller.

The child has been followed for a year and a half since treatment was discontinued. He is developing normally; the mass above the kidney is no longer demonstrable; the bone marrow examination is normal.

One patient with chronic myelogenous leukemia of the Naegli type developed a complete remission during treatment with chloroquine mustard.

Case No. 3. C. L. A., a 67-year-old Negro female, was admitted to the hospital complaining of weakness, fever of two weeks' duration, and progressive swelling of her face and neck.

On physical examination she was found to have diffuse lymphadenopathy, with marked edema of the skin and many areas of nodular infiltration. On biopsy these were shown to be infiltrates of leukemic cells. Her white blood cell count was 13,600, and the differential blood count showed 67 per cent immature myelocytes of the Naegli type. The bone marrow was diffusely infiltrated with similar cells.

This patient was treated with chloroquine mustard intramuscularly, receiving 0.1 mg. kg. each day for one week. Thereafter she was treated every other day and the dose was increased to 0.2 mg. kg. (FIGURE 3). On the thirty-second day she became confused, complained of paresthesias, and developed hallucinations that lasted for several hours. At that time her white blood cell count and differential blood count had returned to normal; the enlarged nodes had decreased progressively; the skin infiltration had disappeared; and the bone marrow examination was normal except for 2 per cent of monocytoid myelocytes. The patient was discharged from the hospital and was well for 6 weeks, when the disease relapsed.

In one newborn infant with so-called acute congenital leukemia of the myelogenous type all evidence of the disease disappeared during treatment and the child has been well, with no evidence of recurrence, for 14 months.

Case No. 4. D. K., an 8-day-old white male, became jaundiced on the fourth day after normal delivery and developed a temperature of 103° F. The platelet count was 7000 per cu. mm., the total white blood cell count was 5050 per cu. mm. The differential blood count revealed 64 per cent blasts and 7 per cent progranulocytes. The spleen was enlarged to the iliac crest, and the liver was also markedly enlarged. The results of the bone marrow examination were considered to be characteristic of acute myeloblastic leukemia by three independent observers.

The child was treated with 0.2 mg. kg. of chloroquine mustard intramuscularly each day for 5 days. The fever subsided within 48 hours after treatment was started. The white blood cell count increased to 12,500, and there was a progressive reduction in the number of blasts over the next 10 days. The platelet count rose to normal values within 2 weeks. The child regained his appetite, started to gain weight, and returned to a state of normal health. He has been followed for a period of 13 months with no evidence of recurrence.

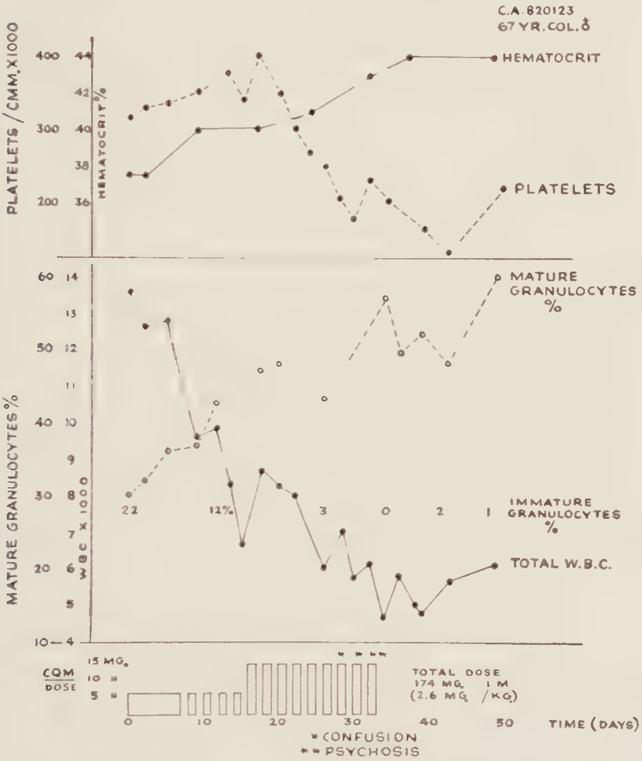

FIGURE 3. Effect of chloroquine mustard (CQM) in a patient with myelo-monocytic leukemia.

In 4 patients there was suggestive evidence of chemotherapeutic activity without the production of full remission. In 1 patient with chronic lymphatic leukemia who received a total dose of 0.7 mg./kg. intravenously, the total white blood cell count fell progressively from 150,000 cu. mm. to 34,000 cu. mm. over a period of 40 days. This was accompanied by an increase in the percentage of neutrophils from 4 per cent to 26 per cent. There was also symptomatic improvement, but no change in the hemoglobin concentration. On the fortieth day the patient was given 10 mg. of triethylene melamine in another clinic, and a complete remission of the disease occurred.

In one child with acute blastic leukemia that was refractory to A-Methopterin, to 6-mercaptopurine, and to steroids, there was a progressive reduction in total white blood cell count from 6500 to 1800 cu. mm., with a progressive reduction in the number of blasts in blood and bone marrow. There was no increase in hemoglobin or platelet count, and the disease progressed rapidly after treatment with chloroquine mustard was terminated.

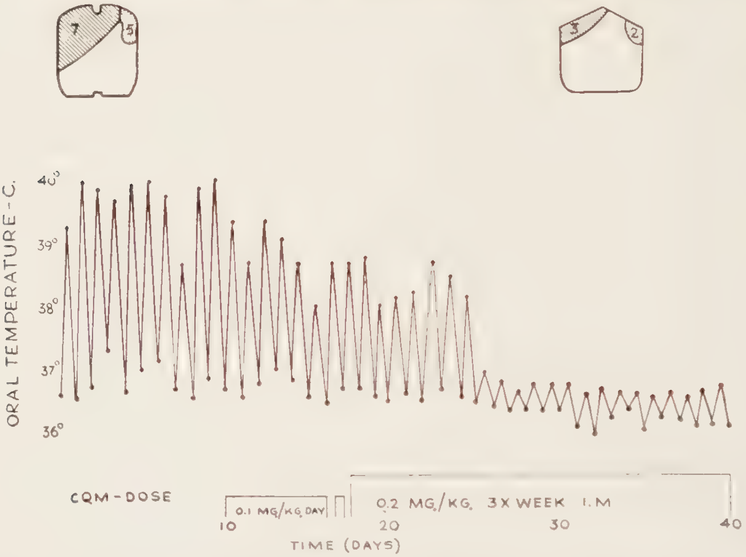

FIGURE 4. Effect of chloroquine mustard in a patient (M. P.) with Hodgkin's disease In the diagram of the abdomen at the top of the chart the size of the liver is indicated by the crosshatched area and the size of the spleen by the stippled area. The numbers in the shaded areas indicate the location of the lower edge of the organ in cm. below the costal margin. The maximal and minimal daily temperatures are shown in the graph.

One patient with Hodgkin's disease showed objective evidence of improvement during treatment with chloroquine mustard (FIGURE 4). There was progressive reduction of the size of the liver and spleen during treatment, fever subsided, and itching abated. In this patient, the disease relapsed 2 weeks after treatment was terminated. In one patient with reticulum cell sarcoma, there was a temporary reduction in the size of a mass of abdominal nodes during treatment, and this was accompanied by symptomatic improvement that lasted approximately 3 weeks.

In none of the other patients in this study was there objective evidence of chemotherapeutic activity. It must be realized that these studies were designed to explore the pharmacology of this agent in man. Definitive evaluation of chemotherapeutic activity will require further study.

QUINACRINE MUSTARD

Method of Study

The drug. Only the HCl salt of quinacrine mustard has been used in these studies. Initially the drug was synthesized by Price and Sen in the Harrison Laboratories of the University of Pennsylvania, and was supplied in bulk. Subsequently it was supplied by the Sterling-Winthrop Laboratories. It was stored in a vacuum desiccator in the cold, and removed only to weigh out the appropriate dose. The drug was dissolved in normal saline for intravenous or intramuscular use. A fresh solution was prepared daily, and the drug was administered promptly after the solution was prepared. For intramuscular administration, the concentration was adjusted so that the volume of each injection was less than 1 ml. For intravenous administration, an appropriate volume of the stock solution was diluted in 500 to 1000 ml. of normal saline or glucose in water and administered by slow drip over a period of 2 to 4 hours.

For studies of oral administration, the drug was weighed into dry gelatin capsules and stored in a vacuum desiccator in the cold. Before the material was used in man the biological activity of each new lot of the drug was determined by Creech and his associates in mice with ascites tumors by the methods described elsewhere in this monograph.[8]

Selection of patients. As described above.

Procedure. As described above.

Pharmacological Effects

Oral administration. Quinacrine mustard has been administered by the oral route to 5 patients in doses of 0.1 to 0.45 mg. kg. day (10 to 40 mg.) for periods ranging from 1 to 92 days (TABLE 3). In every patient the drug produced nausea and vomiting, which usually began 1 to 3 hours after the drug was ingested. In 1 patient, a single dose of 10 mg. produced such severe nausea and vomiting that further treatment was unwarranted. One patient was able to take a daily dose of 20 mg. for only 3 days before it became necessary to terminate treatment because of nausea and vomiting.

Three patients were able to tolerate a daily dose of 0.4 mg. kg. of quinacrine mustard HCl orally for 2 to 3 months. The total dose was 27 to 30 mg. kg. (1.4 to 2.6 gm.). All of these patients experienced some mild to moderate nausea and all vomited on occasion during the treatment period, but these symptoms were of relatively brief duration and were not sufficiently severe to prevent further treatment. No other untoward effects that might have been due to the drug were observed, and there was no evidence of any therapeutic activity. One patient took 30 mg. of quinacrine mustard daily by mouth for 38 consecutive days and 40 mg. daily for the next 46 days. During this period there was no significant change in the peripheral blood cell count or in the bone marrow. He was then given 40 mg. of quinacrine mustard intravenously each day for 8 days, and promptly developed leukopenia (1200 white blood cells/cu. mm.) and thrombocytopenia (44,000 platelets/cu. mm.).

TABLE 3

TOXICITY OF QUINACRINE MUSTARD IN MAN

Patient	No. of days of treatment	Maximum single dose mg./kg.	Total dose mg./kg.	Toxic reactions
Oral administration				
E. G.	1	0.30	0.3	Nausea and vomiting
E. J.	3	0.33	1.0	Nausea and vomiting
F. C.	92	0.40	27.0	Nausea and vomiting
O. L.	63	0.45	30.0	Nausea and vomiting
A. B.	72	0.45	36.0	Nausea and vomiting
Intramuscular administration				
C. A.	8	0.20	1.1	None
F. F.	9	0.20	1.4	Local reaction
G. K.	13	0.20	2.3	None
M. R.	9	0.36	2.2	Local reaction
F. R.	28	0.30	5.1	None
G. R.	36	0.40	8.6	None
K. R.	5	0.50	2.5	Local reaction
A. L. G.	31	0.50	10.5	None
J. M.	43	0.83	23.3	None
A. G.	14	0.90	8.4	Leukopenia
G. A.	29	0.90	16.0	Nausea and vomiting
M. L.	108	0.90	71.2	Leukopenia, local reaction
E. B.	23	1.00	11.0	Leukopenia, local reaction
Intravenous administration				
F. F.	26	0.25	1.5	
C. K.	5	0.30	1.5	Nausea and vomiting
F. O.	7	0.30	2.1	
K. S.	12	0.30	3.6	Nausea and vomiting
A. B.	8	0.45	4.6	Leukopenia, thrombocytopenia
F. C.	15	0.45	4.9	Nausea and vomiting, leukopenia
J. C.	17	0.50	7.7	Leukopenia
W. L.	12	0.55	6.6	
A. G.	19	0.64	9.6	Leukopenia
R. W.	14	0.70	5.9	Nausea and vomiting

Intravenous administration. Ten patients were treated with quinacrine mustard administered intravenously in doses that ranged from 0.1 to 0.7 mg./kg./day (TABLE 3). The duration of treatment varied from 5 to 26 days. All of these patients developed thrombophlebitis of the veins into which the drug was infused. This was usually accompanied by thrombosis, and in 5 patients there was marked edema of the hands and arms that persisted for several weeks after treatment was discontinued. This reaction precluded further therapy in most of the patients because of local pain and lack of available patent veins.

Five of the 10 patients experienced nausea and vomiting at some time during the course of therapy. This usually began 1 to 2 hours after the infusion and lasted for 3 to 4 hours. It was relatively mild in most instances, and inconstant in occurrence.

Five of the 10 patients developed leukopenia, and in 3 of the 5 there was also thrombocytopenia. Leukopenia occurred in 5 of the 6 patients who received a total dose of more than 4.5 mg./kg.

Intramuscular administration. Quinacrine mustard was administered to 13 patients by the intramuscular route in doses that ranged from 0.1 to 1.0 mg./kg., for periods of time that varied from 4 to 108 days of consecutive treatment. The drug produced mild to moderate pain at the injection site in most individuals and, in 5 of the 13, it caused persisting local induration and inflammation. In 2 patients the local reaction was sufficiently severe to contraindicate further therapy.

One patient experienced moderately severe nausea and vomiting, beginning 1 to 2 hours after each of 4 injections of 0.9 mg./kg. None of the other patients experienced gastrointestinal symptoms that could clearly be related to administration of the drug.

Three patients developed leukopenia, and 1 of the 3 developed thrombocytopenia. Two of these patients received a daily dose of 0.9 mg./kg. for more than 1 week, and the third received a daily dose of 1.0 mg./kg./day. In this group there were only 5 patients who received a daily dose of 0.8 to 1.0 mg./kg. for 1 week or more; 3 of the 5 developed leukopenia. There were 8 patients in whom the daily dose did not exceed 0.5 mg./kg./day; none of these patients developed leukopenia or thrombocytopenia.

In those patients who developed leukopenia following intravenous or intramuscular treatment, the effect on the total lymphocytes was greater than the effect on cells of the granulocytic series, although the total number of circulatory leukocytes of each type was reduced. One striking feature of the differential blood cell count was the occurrence of eosinophilia. In all of the 5 patients who received the higher doses of quinacrine mustard intramuscularly, and in all of the patients who received more than 4.5 mg./kg. intravenously, the number of eosinophils in the circulating blood increased to values that ranged from 14 to 34 per cent. The cells were mature eosinophils in all instances. When the percentage of eosinophils increased there was usually a reduction in the percentage of mature neutrophilic leukocytes.

In none of the patients treated with this drug was there any evidence of hepatic or renal toxicity. Serial electrocardiograms in patients on the larger doses were unchanged. No toxic reactions other than those described above were observed.

Preliminary Evidence of Chemotherapeutic Activity

The type of malignant disease that was present in the patients who received quinacrine mustard parenterally, and the changes that occurred in the disease during treatment are summarized in TABLE 4.

TABLE 4

RESULTS OF PRELIMINARY THERAPEUTIC TRIAL OF QUINACRINE MUSTARD IN MAN

Disease	Number of patients	Change in the disease during treatment		
		Marked improvement	Some improvement	No change
Multiple myeloma	6	1	1	4
Chronic leukemia				
Lymphatic	2	—	2	—
Myelogenous	1	—	—	1
Lymphosarcoma	1	—	—	1
Hodgkin's disease	1	—	—	1
Neuroblastoma	2	—	—	2
Medulloblastoma	1	—	—	1
Glioblastoma	1	—	—	1
Astrocytoma	3	—	—	3
Hepatoma	1	—	—	1
Breast Ca	1	—	—	1
Total	20	1	3	16

In 3 patients, changes suggestive of chemotherapeutic activity have been observed.

One patient (M. L.), a 78-year-old white female with far-advanced multiple myeloma, was confined to bed with constant severe pain when treatment was initiated. This patient received increasing doses of the drug intramuscularly over a period of 108 days. The daily dose was increased from 0.1 mg. kg. day to 0.9 mg. kg. day, and was continued at that level for 20 days, until her total white blood cell count fell to 1900 cu. mm. (FIGURE 5). During this period she became free of pain and returned to an ambulatory status; her appetite returned to normal, and she gained approximately 15 lb. in weight. During this period there was a progressive reduction in the number of plasma cells in her bone marrow and the markedly abnormal electrophoretic pattern of her serum protein returned to normal. During a 3-month period after treatment was discontinued her total white blood cell count and platelet count returned to normal and her red blood cell count and hemoglobin concentration increased to normal levels. This patient experienced a complete remission and has remained in complete remission for 13 months.

A second patient with multiple myeloma (A. B.) was treated with a total dose of 2560 mg. (36 mg. kg.) of quinacrine mustard by mouth over a period of 72 days. During this period his weight increased by 19 lb., but there was no objective evidence of improvement in his disease. He was then given 40 mg. (0.45 mg. kg.) of quinacrine mustard intravenously each day for 8 days. This resulted in moderately severe leukopenia and thrombocytopenia that persisted for 3 weeks after treatment was terminated. At this time he experienced marked relief of bone pain. There was a drop of 2 gm. in the concentration of serum globulin and an increase of 1.4 gm. in the serum albumin, and the number of plasma cells in his bone marrow fell from 14 per cent to 8 per cent. Four weeks after treatment was completed this patient developed an overwhelming bronchopneumonia and expired. At this time the disease had relapsed.

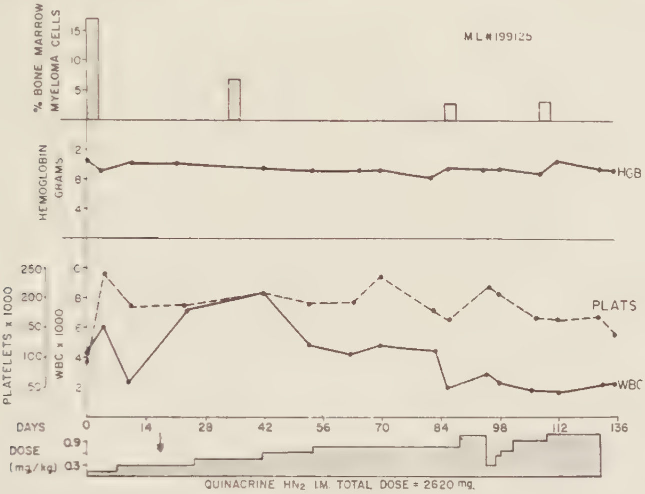

FIGURE 5. Effect of quinacrine mustard administered intramuscularly in a patient with multiple myeloma.

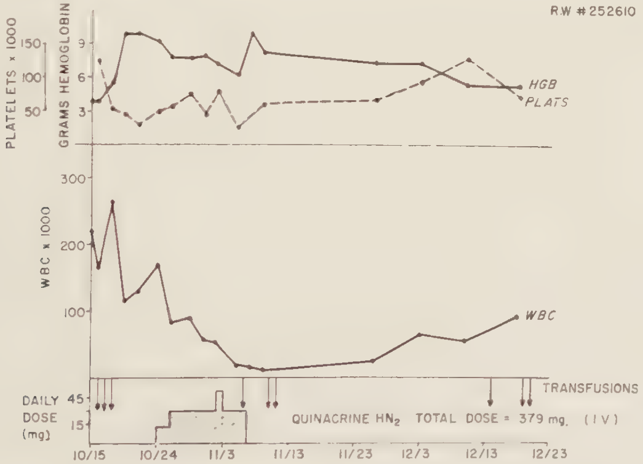

FIGURE 6. Effect of quinacrine mustard in a patient with chronic lymphatic leukemia.

One patient with chronic lymphatic leukemia was treated with 15 mg. of quinacrine mustard intramuscularly for 2 days, followed by 30 mg. intravenously each day for 6 days thereafter. Treatment was discontinued after

8 days because of severe thrombophlebitis. This patient's total white blood cell count had been in the range of 150,000 to 300,000 for 6 weeks prior to the start of therapy. After treatment was started, the total white blood cell count fell progressively from 173,000 cu. mm. on the day treatment was started to 11,000/cu. mm. (FIGURE 6). During this period the number of lymphocytes fell from 96 per cent to 44 per cent and the number of mature neutrophils increased from 4 per cent to 50 per cent. These changes in the peripheral blood were maintained for 4 weeks; then the total white blood cell count started to increase and rose progressively to the previous levels. Despite these changes in the peripheral blood, there was no improvement in the degree of anemia and thrombocytopenia, and there was still considerable infiltration of the bone marrow with lymphocytes at the time when the peripheral blood was essentially normal.

Discussion

Because of methodological difficulties the hypothesis that these agents might effect a selective concentration of the reactive moiety of nitrogen mustard in certain malignant tumors has not been tested. When the agents were found to have chemotherapeutic activity against malignant tumors in experimental animals, we elected to proceed with initial human trial of these two agents. Tissues from many of these patients have been preserved for studies of tissue distribution, and that work is in progress.

These studies have demonstrated that in man the nitrogen mustard analogues of chloroquine and quinacrine have pharmacological effects that are markedly different from those of the parent compounds. Moreover, there are marked differences in the limiting toxic effects of chloroquine mustard and quinacrine mustard. Chloroquine mustard, when administered parenterally, has a striking effect on the nervous system, producing paresthesias, psychosis characterized by hallucinosis and confusion, and convulsions. With quinacrine mustard the limiting toxic effect is bone marrow depression. It will be of interest to determine whether this difference is due to a difference in the concentration of these agents in the two types of tissue. Such studies are in progress.

The fact that severe central nervous system toxicity may occur a short time after a dose of the drug that has been well tolerated for many days indicates that the drug is cumulative in its action and that the reactive moiety probably persists in tissues. This is characteristic of the pharmacological activity of the parent compound, chloroquine.

The fact that chloroquine mustard may have had some therapeutic activity against neuroblastoma is of particular interest in view of the toxicity of this agent to the central nervous system. The evidence of chemotherapeutic activity that was obtained in these studies was sufficient to warrant more detailed study of the effect of this agent on neuroblastoma. Because this tumor is known to undergo spontaneous remission, it is necessary to extend the studies before it can be accepted that the effects observed were, in fact, caused by the drug.

In the other patients in whom there was evidence of chemotherapeutic activity, the changes were not different from those that might be produced by a relatively small dose of nitrogen mustard. When administered intravenously, the toxicity of this compound is greater than that of nitrogen mustard, and the nature of the toxic reaction is similar to that observed with other analogues of nitrogen mustard.[11] It will be of interest to determine whether modification of the substituents of the quinoline ring will reduce the toxic effects on the central nervous system.

It is probable that this compound should not be administered intravenously to man. Our results indicate that it can be administered with safety intramuscularly in doses of 0.1 to 0.3 mg. kg. daily or every other day for periods of 3 to 4 weeks in hospitalized patients under careful supervision.

It is probable that quinacrine mustard HCl is not absorbed in the active form from the gastrointestinal tract. Unless some means can be found to prevent its irritant effect on the vein, intravenous administration is not practicable. The compound can be administered intramuscularly in most patients, even though local reactions are troublesome.

When the drug is administered intravenously, a daily dose of 0.4 to 0.6 mg. kg. for 1 to 2 weeks, or a total dose of 4.5 to 9.6 mg./kg., may produce rather marked bone marrow depression. Somewhat larger doses, for longer periods of time, may be given intramuscularly before bone marrow depression occurs. This suggests that some of the drug may be fixed or inactivated in the muscle.

The evidence that quinacrine mustard has significant chemotherapeutic activity in man is very limited. This may be due to the fact that only a few patients who were treated parenterally received an adequate amount of the drug. The apparent improvement in 2 patients with multiple myeloma suggests that further studies of the effect of the drug in patients with this disease are warranted.

CONCLUSIONS

(1) Studies of the human pharmacology of nitrogen mustard analogues of the antimalarial drugs chloroquine and quinacrine have been conducted in patients with advanced malignant disease.

(2) Chloroquine mustard administered parenterally had as its limiting toxic reaction a profound effect on the central nervous system, manifested by the production of paresthesias, toxic psychosis, and convulsions.

(3) When chloroquine mustard was administered intramuscularly in doses of 0.2 to 0.4 mg. kg., there was preliminary evidence suggestive of some chemotherapeutic activity against neuroblastoma, leukemia, and the solid lymphomas.

(4) Quinacrine mustard produced severe gastrointestinal irritation, and was probably not absorbed in an active form when administered by mouth. When administered parenterally, the limiting toxic reactions were local irritation and bone marrow depression.

(5) In patients who were treated parenterally, there was limited preliminary evidence suggestive of chemotherapeutic activity in two patients with multiple myeloma and in one patient with chronic lymphatic leukemia.

ACKNOWLEDGMENT

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REFERENCES

1. SCHMIDT, L. H. 1946. *In* A Survey of Antimalarial Drugs, 1941–1945. F. Y Wiselogle, Ed. J. W. Edwards, Inc. Ann Arbor, Mich.
2. BERLINER, R. W., D. P. EARLE, JR., J. V. TAGGART, C. G. ZUBROD, W. I. WELCH, N. I. CONAN, E. BAUMAN, S. T. SCUDDER & J. A. SHANNON. 1948. Studies of the chemotherapy of the human malarias. VI. The physiological disposition, antimalarial activity, and toxicity of several derivatives of 4-aminoquinoline. J. Clin. Invest. **27**(Suppl.) : 98–107.
3. WISELOGLE, F. Y., Ed. 1946. A Survey of Antimalarial Drugs, 1941–1945. J. W. Edwards, Inc. Ann Arbor, Mich.
4. PARKER, F. S. & J. L. IRVIN. 1952. The interaction of chloroquine with nucleic acids and nucleoproteins. J. Biol. Chem. **199**: 897–909.
5. IRVIN, J. L., E. M. IRVIN & F. S. PARKER. 1949. Interaction of antimalarials with nucleic acids; acridines and quinolines. Science. **110**: 426–428.
6. JONES, R., JR., C. C. PRICE & A. K. SEN. 1957. The nitrogen mustards related to chloroquine, quinacrine, and pamaquine. J. Org. Chem. **22**: 783.
7. MCNAIR-SCOTT, D. B., & J. S. GOTS. Personal communication.
8. CREECH, H. J. 1958. Discussion: Part II. Ann. N. Y. Acad. Sci. **68**(3): 868.
9. MCKENZIE, D., W. F. DUNNING & R. JONES, JR. Unpublished observations.
10. SCHMIDT, L. H. Unpublished observations.
11. KARNOFSKY, D. A. 1950. Nitrogen mustards in the treatment of neoplastic disease. Advances in Internal Med. **4**: 1–46.

PRELIMINARY CLINICAL EVALUATION OF 1,2-BIS(β-CHLOROETHYLTHIO)ETHANE (SM-1) IN PATIENTS WITH ADVANCED LYMPHOMAS AND NEOPLASTIC DISEASES*

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Recently a sulfur mustard compound, 1,2-bis(β-chloroethylthio)ethane (hereinafter referred to as SM-1), was found by Sassenrath *et al.*[1] to possess a high degree of carcinostatic activity against a variety of mouse ascites tumors. SM-1 was found to be less toxic on a molar basis than nitrogen mustard, but to possess a carcinostatic activity equal to that of nitrogen mustard. In these studies, pretreatment of mice with SM-1 before tumor transplantation demonstrated that SM-1 had a prolonged carcinostatic activity as compared with that of nitrogen mustard. This prolonged carcinostatic activity apparently resulted from the relative insolubility of SM-1 in aqueous solutions. It was concluded by Sassenrath and her co-workers that SM-1 possessed a possible advantage over nitrogen mustard in tumor chemotherapy by virtue of a wider effective dose range and a longer duration of activity within the animals.

The present report deals with preliminary toxicological studies in dogs and clinical results with SM-1 in 61 patients with far-advanced lymphoma and carcinoma.

MATERIALS AND METHODS

The SM-1 employed in this study† was synthesized according to the method of Price and Roberts.[2] SM-1 is a stable white crystalline solid, which is only slightly soluble in water and possesses strong vesicant properties. In cold aqueous suspensions the toxicity and carcinostatic activity were found by Sassenrath *et al.*[1] to remain unaltered over periods of several months. The structural formulas of SM-1, mustard gas, methyl-bis nitrogen mustard, and hemisulfur mustard are shown in FIGURE 1.

Preparation and administration of SM-1. It was found useful to prepare the material as a fine stabilized suspension in serum albumin, since aqueous suspensions of SM-1 were very difficult to administer intravenously because of their rapid rate of settling. The initial preparations employed consisted of a suspension of SM-1 in 20 per cent plasma albumin (stabilized with a drop of Tween 80) that was prepared by grinding SM-1 crystals and lyophil-

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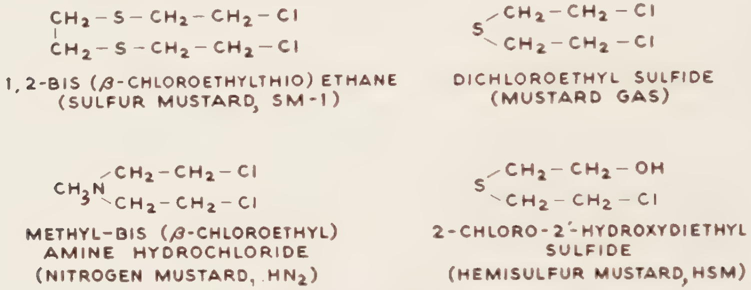
ized human plasma albumin* with mortar and pestle. Normal saline was then added so as to make a suspension with a final concentration of 10 mg./cc. The product was homogenized in a Waring Blendor, giving a stable suspension of fine SM-1 crystals that settled slowly after shaking. The preparations were kept under refrigeration when not in use. This method of preparation was employed until it was found that the mustard slowly lost its potency over a period of several weeks even when kept under refrigeration. Subsequently, SM-1 was prepared immediately before administration by grinding the required dose of SM-1 with 5 mg. of lyophilized human plasma albumin, and suspending the product in 2 to 3 cc. of physiological saline by means of a small syringe. The SM-1 suspensions were administered directly into dogs and patients intravenously through a No. 20 gauge needle without the use of saline infusions.



1, 2-BIS (β-CHLOROETHYLTHIO) ETHANE (SULFUR MUSTARD, SM-1)

DICHLOROETHYL SULFIDE (MUSTARD GAS)

METHYL-BIS (β-CHLOROETHYL) AMINE HYDROCHLORIDE (NITROGEN MUSTARD, HN$_2$)

2-CHLORO-2′-HYDROXYDIETHYL SULFIDE (HEMISULFUR MUSTARD, HSM)

FIGURE 1. Chemical structures of SM-1, related sulfur mustards, and nitrogen mustard.

Toxicological studies of SM-1 were made on adult mongrel dogs before administering the drug to patients. In these studies, the drug was administered as single intravenous doses of 10 mg./kg., 5 mg./kg., and 2 mg./kg. In one animal a dose of 9 mg./kg. was injected intramuscularly. The clinical and hematological effects produced were carefully observed.

The patients selected for this study had far-advanced or rapidly progressing malignancy and had received conventional X-ray therapy and chemotherapy. Initial studies were made on hospitalized patients, but as experience was gained with the drug, many patients were treated as outpatients. The diagnoses in all cases were based on microscopic examination. The diagnosis, sex, age, duration of disease, prior therapy, and general condition at the time of therapy with SM-1 are shown in TABLE 1. The dose of SM-1 employed in patients ranged from 0.05 to 2.0 mg./kg. The agent was administered intravenously at intervals of one month or more. In four patients the drug was given intraperitoneally, and in one intrapleurally. Most patients had frequent blood and bone marrow studies and routine clinical kidney and liver function tests.

TOXICOLOGICAL STUDIES IN DOGS

All studies in dogs were made employing the previously suspended and refrigerated sulfur mustard. Two dogs received SM-1, 10 mg./kg., intra-

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* The human plasma albumin was provided through the courtesy of E. McLain, Cutter Laboratories, Berkeley, Calif.

TABLE 1

SM-1 IN THE TREATMENT OF PATIENTS WITH LYMPHOMA

Case No.	Sex, age	Duration of disease, mo.	Previous therapy		General condition	SM-1 Therapy			Effect	Length of remission, mo.	Outcome after last SM-1 treatment
			X-ray	HN2		Route	Total dose, mg.	Dose, mg./kg.			
Hodgkin's disease											
1	M64	7	+	+	fair	I.V.	40	0.5	fair	1	
		8			fair	I.V.	40	0.5	fair	1	
		12			poor	I.V.	3	0.05	poor	0	
		14			poor	I.V.	3	0.05	none	0	died 1 mo.
2	F9	3	0	0	good	I.V.	15	0.5	good	1	
		4			good	I.V.	20	0.5	good	1	
		5			fair	I.V.	2	0.05	good	2	
		9			fair	I.V.	2	0.05	poor	0	living 7 mo.
3	F23	48	+	+	poor	I.V.	10	2.0	good	1	
		49				I.V.	5	1.0	good	5	
		56				I.V.	4	1.0	fair	0	living 6 mo.
4	M31	36	+	+	poor	I.V.	30	0.5	poor	2	died 3 mo.
5	F28	48	+	+	fair	I.V.	30	0.5	good	5	
		53			fair	I.V.	2	0.05	poor	0	living 5 mo.
6	F21	28	+	+	poor	I.V.	6	0.1	none	—	died 6 mo.
7	F23	38	+	+	poor	I.V.	10	0.5	none	0	died 3 mo.
8	M11	3	0	0	fair	I.V.	2	0.1	none	0	living 2 mo.
Lymphosarcoma											
9	F23		+	0	fair	I.V.	3	0.05	poor	0	
					fair	I.V.	3	0.05	poor	0	living 5 mo.
10	M58	22	+	+	fair	I.V.	3	0.05	fair	7	
		29				I.V.	3	0.05	fair	1	living 2 mo.
11	M40	47	0	0	fair	I.V.	30	0.5	fair	3	living?
12	M45	16	+	0	poor	I.V.	15	0.2	none	0	died 1 mo.
13	M73	4	0	0	poor	I.V.	6	0.1	good	3 wk.	died 1 mo.
14	M68	4	0	0	poor	I.V.	30	0.5	none	0	living 2 mo.
15	F27	47	+	+	fair	I.V.	3	0.05	good	3	living 3 mo.
16	F31	48	+	+	poor	I.V.	2.5	0.05	none	0	died 4 mo.

TABLE 1 (*Continued*)

Case No.	Sex, age	Duration of disease, mo.	Previous therapy		General condition	SM-1 Therapy			Effect	Length of remission, mo.	Outcome after last SM-1 treatment
			X-ray	HN2		Route	Total dose, mg.	Dose, mg./kg.			
Lymphosarcoma (*Continued*)											
17	F73	18	+	0	poor	I.V.	3	0.05	none	0	died 1 mo.
18	M53	4	+	0	poor	I.V.	100	2.0	good	1	died 1.5 mo.
19	M57	2	+	+	poor	I.V.	40	2.0	fair	1	died 1 mo.
Chronic lymphocytic leukemia											
20	M67	120	+	0	poor	I.V.	25	0.5	none	0	died 3 wk.
21	F49	2	0	0	fair	I.V.	15	0.1	poor	0	
		6	+	0	fair	I.V.	3	0.05	none	0	died 2 mo.
22	F50	15	+	0	poor	I.V.	20	0.25	none	0	died 3 mo.
23	M45	3	0	0	good	I.V.	6	0.1	none	0	living 1 mo.
Chronic granulocytic leukemia											
24	F37	33	+	0	poor	I.V.	30	0.5	none	0	died
Acute lymphocytic leukemia											
25	M7	4	0	6MP	poor	I.V.	10	0.5	none	0	died 2 wk.
26	M9	18	0	6MP	poor	I.V.	10	0.5	none	0	died 1 wk.

venously. In one animal acute vomiting and bloody diarrhea developed within a few hours, and the animal was found dead the following day. The other animal expired 5 days after injection without clinical evidence of toxicity other than anorexia. Autopsy revealed pulmonary edema and hemorrhage in the former animal and hyperemia of all the viscera in the latter. Two dogs received SM-1, 5 mg. kg., without clinical signs of acute toxicity, except for anorexia and vomiting in one dog within 6 hours after injection. The most striking effect observed was on the hematopoietic system in both animals, characterized by leukopenia beginning at 4 days, which reached a maximum depression at 7 days, and was followed by a return to normal at 3 to 4 weeks. No significant depression of hemoglobin or platelet levels was observed (FIGURE 2). At 1 and 2 weeks these effects on the peripheral blood were accompanied by marked hypoplasia of the bone marrow, which gradually became normal or hypercellular in appearance by the third and fourth weeks following injection.

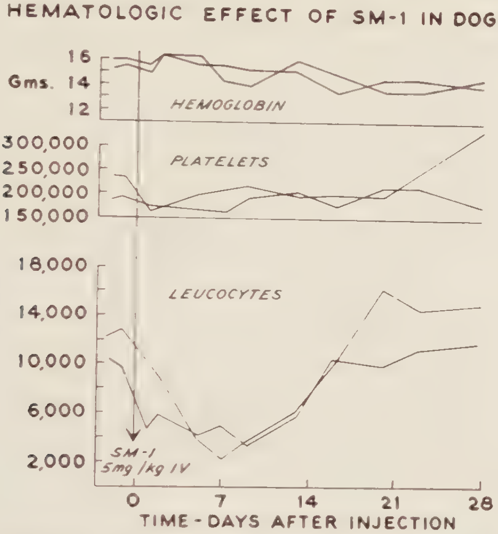


FIGURE 2. Depression of leukocyte count in dogs by SM-1. No significant depression of platelets and hemoglobin was observed.

Two animals received SM-1 at a dosage of 2 mg. kg. without signs of acute toxicity and with only transient leukocyte depression during the first week after injection. One animal was given 9 mg. kg. intramuscularly. In a few hours this dog developed nausea and vomiting and a severe local reaction at the site of injection, characterized by swelling and tenderness and progressing to abscess formation within 2 days. This abscess slowly resolved during the following 2 weeks, and was accompanied by a pronounced leukocytosis which persisted for 10 days.

From these data in dogs it was determined that the acute toxicological and hematological effects of SM-1 closely resembled those produced by nitrogen mustard. No evidence of renal or hepatic damage was clinically detectable in the dogs.

CLINICAL STUDIES WITH SM-1

Since September 1955, sixty-one patients have been treated with SM-1. The dose administered ranged from 0.05 to 2.0 mg. kg., with the majority of patients receiving 0.05 to 1.0 mg. kg. Twelve patients received two or more injections at monthly intervals or greater.

Pharmacological Effects

Local effects. No evidence of venous irritation or thrombophlebitis following direct intravenous injection of SM-1 was observed; this made it possible to give SM-1 to ambulatory patients. It was not found necessary to employ saline infusions and heavy sedation. On rare occasions, when traces of SM-1 were inadvertently deposited outside the vein, a painful local inflammatory reaction developed within 24 hours. In 2 patients the local inflammation was followed by sloughing of tissue and ulceration in the injected area which slowly healed over a 2-week period.

Systemic effects. Anorexia, nausea, and vomiting occurred in most patients 3 to 9 hours after injection. These effects decreased progressively in severity and incidence during the next 24 hours. Diarrhea was not produced. It was our impression that the intensity of nausea and vomiting produced by SM-1 was much less than that resulting from nitrogen mustard. Most patients received oral or intramuscular doses of 25 to 50 mg. chlorpromazine at the time of, and at 2 hours following, SM-1 injection; this materially decreased the gastrointestinal symptoms. A generalized maculopapular eruption over the face and trunk developed in one patient a week after receiving SM-1. The eruption responded to antihistaminic therapy within 48 hours. Two patients developed alopecia at 4 to 6 weeks following recovery from septicemia that developed as a complication of severe hematological depression. No evidence of neurotoxicity was seen in any of the patients who received SM-1.

Hematological effects. The most serious and potentially limiting toxic effect of SM-1 observed was on the hematopoietic system. The hematological response to SM-1 was found to resemble closely that which is produced by nitrogen mustard. Lymphocytopenia resulted within 2 to 3 days following injection and was followed by a decrease in granulocytes by the end of the first week. The maximum degree of leukopenia occurred during the second and third weeks, with recovery by the end of the fourth week. Thrombocytopenia developed in most patients, with the maximum depression occurring at 2 to 3 weeks (FIGURES 3 and 4). Moderate erythrocyte and hemoglobin depression was observed in most patients. In most patients studied, the period of pancytopenia produced by SM-1 lasted approximately 3 to 4 weeks. Serial studies of the bone marrow were made at weekly intervals in a number of patients. Within 7 days hypoplasia of all marrow elements was found with all doses of SM-1 employed. This condition persisted during the second week and was followed by intense marrow regeneration

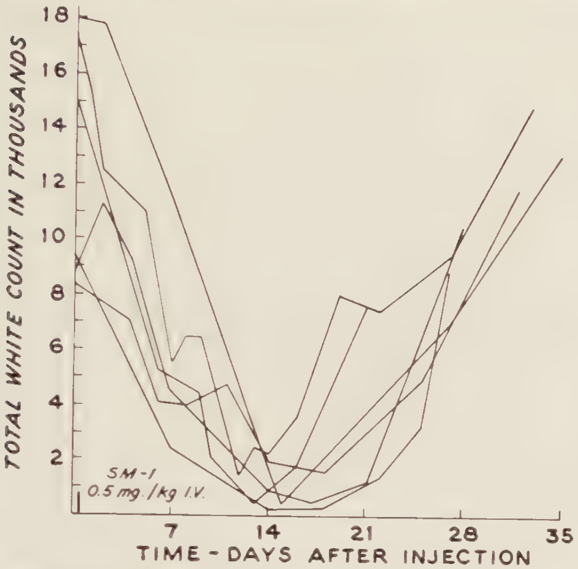


FIGURE 3. Effect of SM-1 upon total leukocytes in patients with lymphomas and carcinomas.

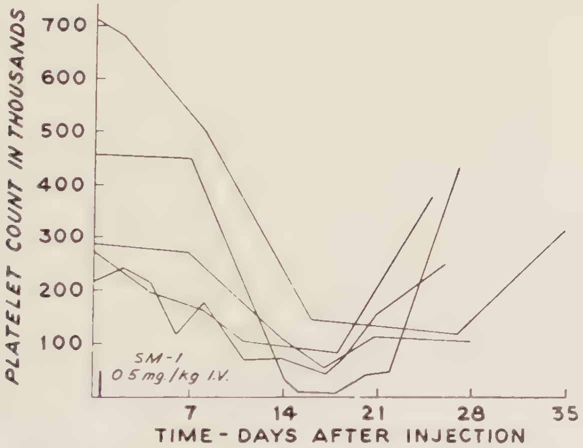


FIGURE 4. Effect of SM-1 upon platelet counts in patients with lymphomas and carcinomas.

and a return to normal cellularity by 4 weeks. Eight patients with lymphoma developed severe leukopenia with counts of less than 500 cells per cu. mm., anemia of less than seven gm. per cent, severe thrombocytopenia, prolonged bleeding and clotting times, cutaneous petechiae, and ecchymosis. Four patients developed septicemia. The most severe hematopoietic depressions occurred in patients with far-advanced disease who were in poor clinical and hematological condition prior to therapy.

Other effects. Repeated studies made on urine, on liver function, and on electrolyte concentration before and after treatment disclosed no evidence of abnormality that could be attributed to SM-1 toxicity.

Therapeutic Effects

Hodgkin's disease. Five out of 8 patients with Hodgkin's disease who received SM-1 (TABLE 1) experienced within a few days a significant subjective improvement characterized by a feeling of well-being and increased appetite. Significant regression in the size of peripheral and mediastinal lymph nodes and in hepatosplenomegaly was observed in 4 patients during the first 2 to 4 weeks following injection. Regression of adenopathy was accompanied by a decrease in such signs of systemic toxicity as fever, pruritis, and fatigue. The prompt decrease of fever with SM-1 therapy in patient No. 1 with abdominal Hodgkin's disease is shown in FIGURE 5. Patient No. 3 had regression of cervical lymphadenopathy and experienced complete and sustained relief from generalized pruritus for the first time in eight months (FIGURE 6). Regression of mediastinal adenopathy in patient No. 5 is shown in FIGURE 7. The durations of clinical remissions produced by SM-1 are recorded in TABLE 1. These ranged from 1 to 5 months, the majority of remissions lasting approximately 1 month. One patient (No. 5) had a complete remission of her disease for 5 months. Three patients received repeated courses of SM-1 with continuing therapeutic effect. Three patients showed no response to SM-1 therapy.

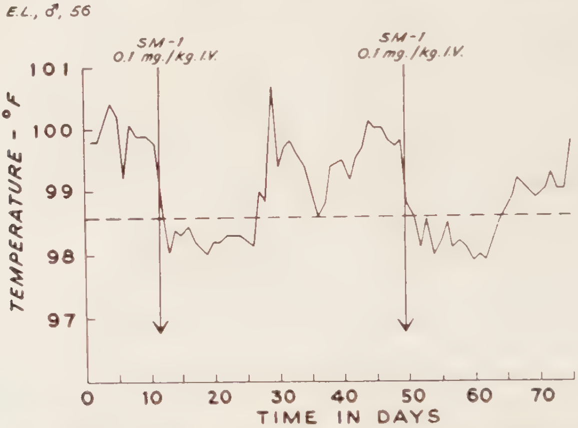


FIGURE 5. Case No. 1. Hodgkin's disease. Demonstrates prompt decrease in fever as a result of SM-1 therapy.

All patients with Hodgkin's disease treated with SM-1 developed a significant degree of bone marrow depression and pancytopenia. A hemolytic streptococcal septicemia resulted in patient No. 5 ten days following injection of SM-1. This patient was successfully treated with antibiotics, blood transfusions, and supportive care until hematological recovery occurred 3 weeks later. Patient No. 3 became pregnant and during the eighth week of pregnancy developed a significant clinical relapse requiring systemic chemotherapy. A dose of 1.0 mg./kg. SM-1 resulted in regression of clinical symptoms and of lymphadenopathy and in expulsion of the fetus 2 weeks later.

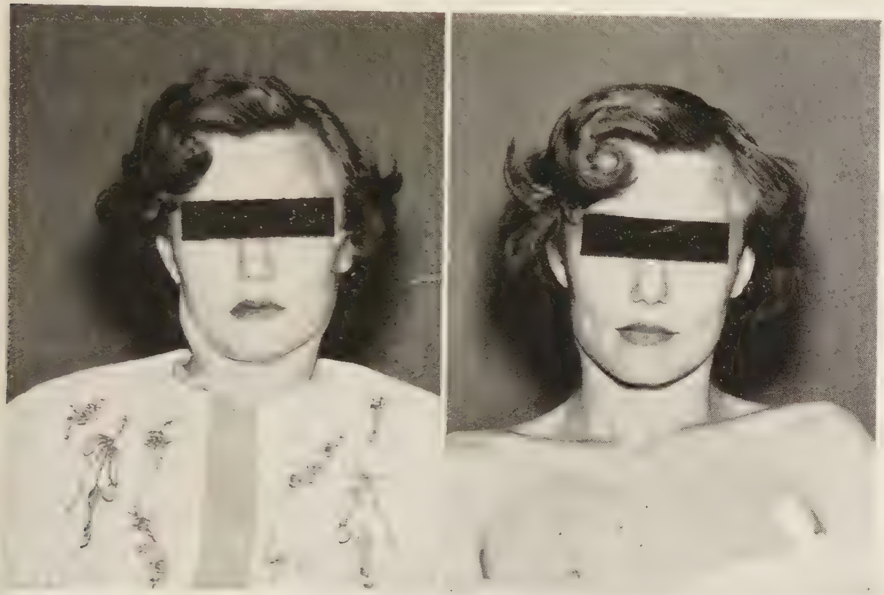


Figure 6. Case No. 3. Hodgkin's disease. Showing regression of cervical adenopathy and amelioration of constitutional symptoms following SM-1 therapy. Photograph taken one month after therapy.

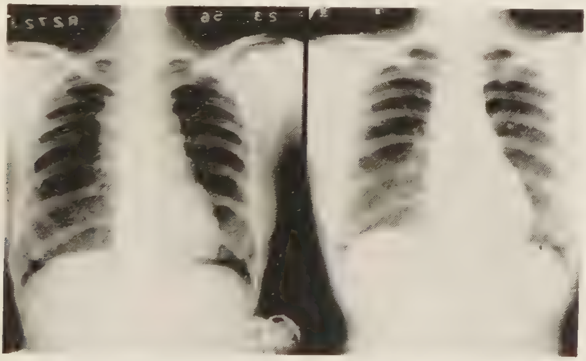


Figure 7. Case No. 5. Hodgkin's disease. Regression of mediastinal adenopathy following SM-1 therapy.

Lymphosarcoma. Eleven patients with lymphosarcoma were treated with SM-1 (table 1). Five showed a good or fair response to therapy characterized by a feeling of well-being, increased appetite, and definite regression of adenopathy and hepatosplenomegaly within 1 to 3 weeks following treatment. The longest period of regression observed was 7 months, the majority of remissions lasting approximately 1 month. Five patients had no response to treatment with SM-1. Patient No. 13 showed a striking regression of generalized lymphadenopathy and hepatosplenomegaly within 1 week following 0.1 mg./kg., but died 19 days later with hemolytic staphylococcus septicemia and severe bone marrow depression (FIGURE 8).

Chronic leukemia. Four patients with chronic lymphocytic leukemia received SM-1 (0.1 mg./kg., 0.25 mg./kg., and 0.1 mg./kg., respectively), with no significant effect upon the clinical findings and hematological status of the patients. One patient with chronic granulocytic leukemia in a moribund stage received 0.5 mg./kg. The patient died 5 days later with a profound pancytopenia. No regression of splenomegaly occurred during this period.

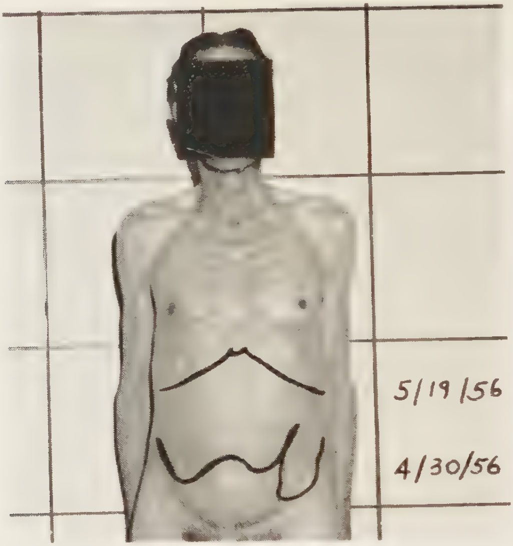


FIGURE 8. Case No. 13. Lymphosarcoma. Showing dramatic regression of massive hepatosplenomegaly over a 19-day period following SM-1, 0.1 mg./kg.

Acute lymphocytic leukemia. Two children in the terminal phase of acute lymphocytic leukemia received doses of 0.5 mg./kg. without significant clinical or hematological effect.

Other neoplastic diseases. Thirty-five patients with neoplastic diseases other than lymphomas received SM-1. The diagnoses included: bronchogenic carcinoma 13, carcinoma of the ovary with carcinomatous ascites 4, carcinoma of the breast 4, malignant melanoma 1, choriocarcinoma of the testis 2, Ewing's tumor 1, adenocarcinoma of the kidney 2, teratoma 1, carcinoma of the parotid 1, carcinoma of the pancreas 1, carcinoma of the palate 1, metastatic anaplastic carcinoma (primary site undetermined) 4, and thymoma 1. Favorable effects with SM-1 were observed in only 3 patients. One patient with bronchogenic carcinoma had a decrease in hilar tumor mass with an improvement in atelectasis, a decrease in pleural effusion, and a marked regression of a supraclavicular tumor metastasis lasting for 3 weeks. Two other patients with bronchogenic carcinoma experienced transient decrease in dyspnea, with slight decreases in tumor size as determined by serial roentgenograms. One patient with generalized carcinoma of the breast and malignant pleural effusion had a complete disappearance of pleural effusion over a period of 4 weeks following 0.05 mg./kg. SM-1 intravenously (FIGURE 9).

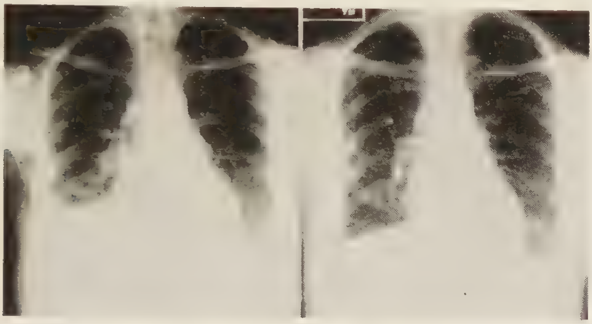


FIGURE 9. Carcinoma of breast. Showing disappearance of pleural effusion in right chest over a 4-week period following SM-1 therapy.

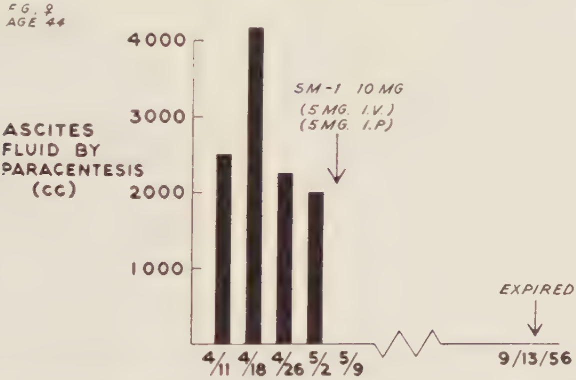


FIGURE 10. Carcinoma of ovary with ascites. Shows prompt and complete suppression of carcinomatous ascites following simultaneous intraperitoneal and intravenous therapy with SM-1.

Four patients received SM-1 intraperitoneally for carcinomatous ascites. These included 2 with carcinoma of the ovary, 1 with carcinoma of the breast, and 1 with anaplastic carcinoma, site or origin undetermined. Within 24 hours after injection of 5 mg. of SM-1 two of these patients developed peritoneal irritation that persisted for 3 to 4 days and was accompanied by paralytic ileus and diffuse abdominal tenderness. An objective therapeutic response was seen in one patient with carcinoma of the ovary who during the preceding month had required weekly paracenteses of over 2000 cc. of ascitic fluid. A prompt and complete disappearance of ascites followed subsidence of the peritoneal reaction, and no further paracenteses were required during the remainder of her illness (FIGURE 10). The suppression of ascites was accompanied by a good subjective clinical remission for 3 months before the patient relapsed and died. Complete replacement of the peritoneal cavity with tumor tissue was found at autopsy.

One patient with carcinoma of the ovary with pleural effusion received 2 mg. SM-1 intrapleurally without local reaction, systemic toxicity, or effect on the formation of the effusion.

Discussion

The results of the present investigation with SM-1 in patients with advanced lymphoma and carcinoma demonstrate that the toxicological and therapeutic spectra of SM-1 are very similar to those of nitrogen mustard. SM-1 produces acute gastrointestinal toxicity within 24 hours following injection, followed by marked hematopoietic depression involving leukocytes, platelets, and erythrocytes. Hematological depression persists for 2 to 3 weeks; the blood picture returns to normal at approximately 4 weeks. The toxicity of SM-1 for the hematopoietic system appears to equal or exceed that produced by nitrogen mustard. Further clinical studies will be necessary to determine the optimal therapeutic and least toxic dose of SM-1.

It is interesting to note that, despite lower doses per kg. body weight, the magnitude of hematopoietic depression produced was much greater in patients than in dogs. It is possible that the SM-1 produced more severe marrow toxicity in man as a result of the generally poor clinical and hematological status of the patients employed in these early clinical trials. A possible advantage of SM-1 over nitrogen mustard is its ease of administration. It is not necessary to employ intravenous infusions such as are employed for nitrogen mustard, since SM-1 does not produce pain or thrombophlebitis when injected intravenously. As a result, the drug can be employed in ambulatory patients in the outpatient clinic. The degree of nausea and vomiting produced by SM-1 appears to be less than that produced by nitrogen mustard, and can be partially controlled by oral or intramuscular doses of chlorpromazine.

Varying degrees of objective therapeutic response were observed in approximately half of the patients with Hodgkin's disease and lymphosarcoma who received SM-1. These effects consisted of regression in size of lymph nodes, reduction of size of the liver and spleen, weight gain, decrease in fever, relief of pruritus, and a feeling of well-being. In most patients the duration of response was brief, lasting only 4 or 5 weeks; however, several patients had more prolonged periods of clinical remission. Only minimal and insignificant effects were seen in other types of lymphoma and carcinoma studied. Of the latter group, one patient each with bronchogenic carcinoma, carcinoma of the breast, and carcinoma of the ovary with abdominal carcinomatosis showed a significant therapeutic response, consisting of reduction in size of tumor mass or reduction of pleural effusion or ascites.

Although SM-1 was found by Sassenrath and her associates[1] to have a prolonged carcinostatic effect in mice as compared with nitrogen mustard, no clinical evidence of prolonged toxicity or therapeutic effect was observed in the patients treated. Toxic effects other than on the gastrointestinal tract and hematopoietic system were observed in a few patients. These included alopecia, maculopapular skin eruption, and fetal death. No evidence of neurotoxicity such as has been reported for hemisulfur mustard[3] was observed in the patients treated with SM-1.

On the basis of the present studies with SM-1 it is felt that, aside from its technically more simple administration, SM-1 possesses no chemotherapeutic advantage over nitrogen mustard and may possess greater toxicity for the hematopoietic system. Further studies will be necessary to determine its relative effectiveness in earlier stages of Hodgkin's disease and lymphosarcoma and in the palliative treatment of patients with carcinomatous effusions and ascites.

Summary

The sulfur mustard compound 1,2-bis(β-chloroethylthio)ethane (SM-1) has been evaluated for toxicity in dogs and has been employed in preliminary studies on 61 patients with advanced lymphoma and carcinoma. The clinical response and toxicological effects of SM-1 closely resemble those produced by nitrogen mustard. Objective clinical responses were seen in Hodgkin's disease and lymphosarcoma and rarely in carcinomas.

Acknowledgment

The authors express their appreciation to Barbara Hughes and Marie Doherty for their technical assistance in the study described in this article.

References

1. Sassenrath, E. N., D. C. Morrison, A. M. Larson & D. M. Greenberg. 1950. Evaluation of the carcinostatic activity of 1,2-bis(beta-chloroethylthio)ethane. Cancer Research. **15**: 755.
2. Price, C. P. & R. M. Roberts. 1947. Some reactions of 1,2-bis(beta chloroethylthio)-ethane. J. Org. Chem. **12**: 255.
3. Rutenburg, A. M. & A. M. Seligman. 1956. Palliation of carcinomatosis with ascites by means of hemisulfur mustard (2-chloro-2′ hydroxydiethyl sulfide). New Engl. J. Med. **255**: 361.

CLINICAL OBSERVATIONS WITH 1,6-BIS(β-CHLOROETHYLAMINO)-1,6-DEOXY-D-MANNITOL DIHYDROCHLORIDE (BCM) IN MALIGNANT DISEASES

By Camillo Sellei and Sandor Eckhardt
National Oncological Institute, Budapest, Hungary

Introduction

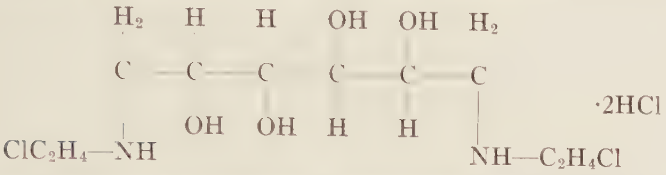
The past ten years have witnessed great advances in research on the chemotherapy of malignant tumors. The facts that cancer is the second leading cause of death and that malignant diseases of the hemopoietic system are gaining more and more ground urge us to increase our endeavors to improve the therapy of these disorders.

Research on these problems has been underway in Hungary since 1935. Jány, Sellei, and Németh[1] have shown that mustard gas has a considerable inhibiting effect upon the metabolism of tumor tissues. Later Gilman and Philips[2] summarized the literature on nitrogen mustard, a compound of lower toxicity than mustard gas. Clinical researches have been made with this latter compound.[3, 4] Mustard gas has proved to be effective in the treatment of lymphogranulomatosis and related diseases. Its disadvantages are its considerable toxicity and its lack of consistent therapeutic effects on carcinomas and sarcomas.

Successes achieved by nitrogen mustard have inspired many investigators to attempt to synthesize compounds of similar therapeutic activity with decreased toxicity. Several medicaments put into circulation between 1946 and 1956 have answered these requirements to a certain degree.

There are several reports[5-8] of trials with aromatic nitrogen mustard derivatives, but satisfactory results were not obtained. β-Chloroethyl groups have been bound to amino acids. Larionov's[9] "sarcolysin" produced notable effects. Following the investigations of Druckrey[11] and Ishidate,[12] the *N*-oxide mustard preparations have gained considerable ground, and the drugs released as Nitromin and Mitomen have also been found useful in our clinical practice.[13]

Regarding researches in Hungary, the investigations made with Magyar's compounds, as described by Masszi and his collaborators,[14] should be mentioned first. No results have been obtained as yet with the piperidine derivatives. Experiments on a larger scale were initiated by the Hungarian Pharmaceutical Research Institute in Budapest, in the course of which several sugar-mustard derivatives were produced. One of these, synthesized by Vargha and Dumbovich,[15-17] deserves special attention: 1,6-bis(β-chloroethylamino)-1,6-deoxy-D-mannitol dihydrochloride, abbreviated as BCM and bearing the commercial name of Degranol. The structural formula of this compound is



$$\begin{array}{cccccc} H_2 & H & H & OH & OH & H_2 \\ C & -C- & C & C & -C- & C \\ | & OH & OH & H & H & | \\ ClC_2H_4-NH & & & & & NH-C_2H_4Cl \end{array} \cdot 2HCl$$

This paper presents a summary of the favorable results obtained with the above compound in our clinical practice during the period from March 1, 1955, to February 28, 1957.

Effect of BCM on Animal Tumors

BCM dissolves in water easily. It is a tasteless, odorless white powder distributed by the Chinoin Factory in powder ampoules under the commercial name Degranol. In clinical use it is expedient to dissolve it in physiological saline, and it is stable in aqueous solution. One ampoule contains 50 mg. BCM.

Kellner, Németh, *et al.* [18-21] have carried out laboratory studies on BCM. In collaboration with other research workers (K. Lapis, L. Holczinger, J. Sugár, T. Gáti, and others) they found the LD_{100} on rats to be 160, on mice 200, and on guinea pigs 100 mg./kg. administered intravenously. These data show a lower toxicity than nitrogen mustard. BCM's local irritating effect upon animal tissues is relatively small, although irritation has been observed in man. The effects of BCM injected intravenously and intraperitoneally have been studied in different animal tumors (for example, M1 rat sarcoma, Ehrlich ascites carcinoma, Guerin carcinoma, and Crocker sarcoma 180), and marked inhibitory effects could be obtained. In a series of interesting experiments, Lapis and Németh have found that BCM effectively inhibits the production of metastases.

While Kellner has shown the inhibitory effect of BCM upon tumors, Németh has made researches from the hematological point of view. In mice inoculated with leukemia of the AKR-Paterson strain, upon treatment with 6 mg./kg. doses, neoplastic growth disappeared completely and there was a considerable fall in the leukocyte count. This effect of BCM on the blood count is mediated through the bone marrow. The lymphatic system was found to be the most sensitive, and less severe effects were observed on myelo- and erythropoiesis. Fibrosis, hemosiderosis, and reticulosis were observed in the involuted lymph nodes. Obvious morphological changes could be found in the single cells.

Effects of BCM in Man

Because of the promising results in animals, BCM was considered for clinical trial in patients with neoplastic diseases. In the first trial of the new drug small doses were used in patients with far-advanced cancer. Only after the dosage was worked out did we begin to treat a greater number of patients suitable for therapeutic evaluation.

We wish to draw attention to the difficulties in evaluating clinical experiences. Most patients simultaneously require other treatments (for example, transfusions, hormones, vitamins), and these definitely interfere with the accumulation of clear-cut experimental data. On the other hand, supportive treatment is of vital interest to the patients. Nor can different patients suffering from the same disease be considered together; most of them, especially those with malignant hematological diseases, have a history of various previous treatments. Taking all these circumstances into consideration, the following topics will serve as an outline for this report: (1) methods of drug administration; (2) dosage; (3) duration of treatment; (4) effects of BCM upon normal tissues; (5) effects of BCM upon neoplastic diseases of the blood-forming organs; and (6) effects of BCM upon malignant tumors.

METHODS OF ADMINISTRATION

We decided to inject BCM intravenously. The injections had no immediate effects. We generally used physiological saline as a diluent. Szentklárai,[22] however, on the basis of other considerations, has used BCM dissolved in 1000 mg. of vitamin C solution. In aqueous solution BCM's full potency is retained for at least 24 hours, but it begins to decrease after 48 hours. BCM should not be injected intramuscularly or subcutaneously. When BCM solution extravasates, poorly demarcated areas of necrosis are sometimes seen. It may be safely administered into any pleural or peritoneal effusions. So far no effects have been observed when it is administered by mouth.

The smallest effective dose is 0.8 to 1.0 mg. kg., and there is no reason for giving a smaller dose. The largest dose given to man so far has been 10 mg. kg. (500 mg. given to a man weighing 50 kg.). Many factors must be considered in determining the dosage for individual patients. Different diseases react differently, and thus the size of a single dose as well as the total amount used in the course of a treatment should vary according to the nature of the case as well as according to the individual susceptibility of the patient. The effective dosage is also influenced by the amount of previous treatment with cytostatic agents. Favorable effects have been obtained by daily dosages of 1 to 2 mg. kg., to a total of 800 to 1300 mg. in a course of the treatment.

We found that the most favorable results could be obtained when BCM was injected on alternate days. An exception is acute leukemia, in which daily injections yield better results. We shall revert to this question later when discussing resistance and cumulative toxicity.

In the normal individual BCM produces leukopenia, but this effect occurs later than in patients suffering from systemic disorders. Leukopenia generally appears 6 days after the administration of a single large dose of BCM. This reaction, however, may be prevented by giving 20 units of ACTH daily. A healthy, hematologically intact individual does not develop leukopenia from a single 150-mg. dose of BCM. Leukopenia occurs earlier in children. This may be explained, perhaps, by the physiological lymphocytosis and the extreme sensitivity of the lymphatic elements in childhood. In the leukopenia produced by BCM in healthy subjects there is a decrease in the number of lymphocytes and eosinophils.

The bone marrow depressant action of BCM has been studied with special care in patients with neoplastic diseases of the blood-forming organs. BCM had the most severe effect on elements of the lymphatic system, a smaller effect upon the thrombocytes and myeloid system, and a slight effect on erythropoiesis. Bone marrow aspirations showed the number of cellular elements to be decreased in the same order as above; the younger cells were found to disappear first, proving that they were more sensitive to BCM than were the more mature cells.

We are now studying the dynamics of the bone marrow depression, but the results are too extensive to present here. There is no doubt that leukemic cells are more sensitive to BCM than normal cells, and this explains the therapeutic effects of BCM.

BCM may exert some adverse effects on the liver. It does not alter the composition of the serum proteins, and liver function tests are not adversely affected during the course of treatment. Sometimes, however, slight jaundice and symptoms of chronic liver dysfunction occur long after treatment, mostly in patients who are in poor condition and who have received very large doses. A patient presenting this complication will be discussed later.

BCM has no consistent effects on liver function, on the blood sugar, or on the nonprotein nitrogen levels in the blood. The serum uric acid value may rise in the course of the treatment. No physiological disturbances have been observed: circulation, blood pressure, respiration, and EKG remain unchanged.

There are only sporadic data concerning the effects of BCM on the endocrine system. Several female patients report absent or delayed menstruation during treatment. We treated one patient who bore her child to term and had no disturbances throughout her pregnancy. We noted no disturbances in libido or potency. There is some evidence that the adrenal gland was affected, since symptoms of weakness connected with nausea and leukopenia always improved when treated with cortisone, whereas adrenocorticotropic hormone (ACTH) seldom relieved these symptoms. Even very large doses did not produce abnormal nervous or psychic effects.

THERAPEUTIC INDICATIONS

BCM has been used in the treatment of 164 patients with various disorders of the hematopoietic and lymphatic systems (TABLE 1).

One-hundred and fifty-four patients with malignant tumors were treated with BCM. The number of patients suffering from the various kinds of cancer is shown in TABLE 2.

The total number of patients treated was 318. In addition to these, BCM was tried in some dermatological cases (for example, psoriasis), but we cannot report any favorable results, except in some cases of mycosis fungoides. Trials are in progress in polycythemia vera.

LEUKEMIAS

The effects of BCM upon various forms of leukemia are summarized in TABLE 3.

TABLE 1

	Number of cases treated
Chronic lymphocytic leukemia	35
Chronic myelocytic leukemia	18
Acute paramyeloblastic leukemia	10
Lymphosarcoma	17
Reticulum cell sarcoma	7
Hodgkin's disease (lymphogranulomatosis)	59
Multiple myeloma, plasmocytoma	8
Giant follicular lymphosarcoma (Brill-Symmers)	9
Reticulosis	1
Total	164

TABLE 2

Diagnosis primary site	Number of cases treated
Bone	7
Lung	54
Breast	13
Ovary	8
Uterus	5
Parotid (pleomorphic adenoma with metastases)	2
Esophagus	1
Stomach	13
Large bowel	3
Rectum	7
Pancreas	3
Gall bladder	1
Larynx	2
Thyroid	1
Tonsil	2
Malignant tumor of mediastinum	4
Kidney	1
Seminoma testis	3
Prostate	1
Melanoma	2
Retinoblastoma	4
Glomus tumor	1
Meristoma	1
Unknown primary tumor with metastases	9
Others	2
Basal cell carcinoma	4
Total	154

TABLE 3

Diagnosis	Number of cases	No response	Free of symptoms for			
			3	6	12	24 months
Chronic lymphocytic leukemia	35	4	31	27	25	17
Chronic myelocytic leukemia	18	4	14	9	6	5
Acute paramyeloblastic leukemia	10	5	5	2	—	—

Chronic Lymphocytic Leukemia

The best results were obtained in cases of chronic lymphocytic leukemia (TABLE 3). The majority of the patients have shown improvement, and more than 50 per cent of the responsive cases are completely free of symptoms 2 years after initiation of BCM therapy. Many of them have no signs of lymphocytic leukemia, and the bone marrow and blood pictures appear normal.

Case history No. 1. In March 1955 R. C. received 100 mg. on alternate days to a total dose of 800 mg. On admission his leukocyte count was 19,600, with 78 per cent young lymphocytes. As a result of the treatment his leukocyte count fell to 9600, with 34 per cent lymphocytes, he gained 5 kg. weight, and his dermatological symptoms improved. Bone marrow aspiration made in another institute 2 years later was normal. He has no complaints, and no changes have occurred in his blood picture since his discharge.

It is noteworthy that patients who had received X-ray treatment for a long time and finally became refractory have improved on BCM.

Case history No. 2. W. R., a 68-year-old man, was treated for 4 years with radiotherapy. He was completely refractory to X-ray treatment and had apple-sized cervical and axillary nodes, WBC 100,000, and 90 per cent lymphocytes. After a treatment with 900 mg. BCM the WBC dropped to 6000, and the lymphocytes disappeared. He did not need another treatment until a year later, and then his condition was again restored to normal.

In addition to its effect on the hematopoietic system BCM caused some of the complications of leukemia to disappear; annoying leukemic infiltrations covering the whole body vanished, and pyodermic processes were cured.

BCM in comparatively small doses may have favorable effects. Dramatic results are seen chiefly after 300 to 400 mg., and larger doses of 800 to 900 mg. are seldom needed. A decrease in the number of the lymphocytes can generally be observed simultaneously with the drop in the WBC at the third injection, on the sixth day of the treatment. However, the blood picture often shows improvement after the first injection. This clearly shows the importance of careful dosage control. The duration of response is about 1 to 2 years. Cells with large bizarre nuclei and with the loose chromatin structure of young lymphocytes then appear again and flood the blood. Treatment initiated at this phase usually prevents relapse. Our fatalities were all over 60 years old, and 2 of them died as a result of complications.

Chronic Myelocytic Leukemia

Favorable results were obtained also in cases of chronic myelocytic leukemia. Five of 18 patients remained asymptomatic for more than 2 years after treatment. In these cases, without exception, more mature myeloid elements predominated. Against the less mature promyelocyte type of cells, large doses of BCM (total dosage in the range of 1400 to 1800 mg.) may be effective. Such treatment, of course, may involve severe toxicity to normal hematopoiesis already severely damaged by the leukemic process. These facts may explain why chronic myelocytic leukemia rarely responds satisfactorily to BCM. Nevertheless, we are able to report a symptomless period of approximately 6 months in about half of our patients. We attach great importance to simultaneous treatment with other drugs such as Myleran or Colcemid; this combination therapy is currently being investigated.

In cases where favorable results were obtained, these appeared after a total dose of approximately 700 to 800 mg. BCM. First, the number of myeloblast-promyelocyte elements decreased in the bone marrow; later, increasing numbers of degenerated myelocytes were observed. Younger eosinophils are especially sensitive, and they are rarely found in the bone marrow under treatment. Much greater resistance is shown by the basophil cells. Bone marrow regeneration begins only about 8 weeks later. Splenomegaly rarely responds to treatment with BCM.

Acute Leukemia

Our observations on acute leukemia are worth noting. Table 3 indicates that we obtained poor results with BCM. Only 2 of 10 patients were symptomless over a period longer than 6 months, and these patients died.

Case history No. 3. Mrs. J. S., 49-year-old female, had shown no improvement upon the usual treatment with ACTH + transfusion of blood + Purinethol(6-mercaptopurine). We then gave her 100 mg. BCM daily for 15 days, to a total of 1500 mg. This treatment resulted in notable changes. The paramyeloblasts previously present in the blood picture and in the bone marrow disappeared. An initial leukocyte count of 22,000 decreased to 800, and no more abnormal cells could be observed. Complete bone marrow regeneration began after 2 weeks, and the patient was discharged. After 3 months she relapsed and this time was resistant to all treatment.

Case history No. 4. The same type of treatment was not effective in Sz. E. A complete exhaustion of the bone marrow ensued after treatment with 1400 mg. BCM. Abnormal cells disappeared, and histological examination did not reveal any signs of leukemia. Death occurred as a result of massive hemorrhage.

Many of our patients could be dismissed for periods of 3 to 4 months in partial hematological remission. We gave these patients BCM on alternate days. In 5 cases the normal course of the illness was not changed.

In summary, BCM is superior to other chemotherapeutic agents in chronic lymphocytic leukemia, it is equal to other drugs in chronic myelocytic leukemia, and it has dramatic effects in some cases of acute leukemia.

LYMPHOMAS

Hodgkin's Disease

BCM has proved effective in Hodgkin's disease (TABLE 4). Twenty-two of 59 cases remained symptomless for more than 2 years. When relapse occurred the patients usually responded to a second course of treatment. The 13 patients who died within 6 months suffered in most cases from Hodgkin's sarcoma. Large doses were necessary and, in 5 cases, because of previous treatment with cytostatic agents (nitrogen mustard, actinomycin C), it was necessary to interrupt treatment for fear of severe bone marrow depression.

TABLE 4

Diagnosis	Number of cases treated	Free of symptoms for			
		3	6	12	24 months
Hodgkin's disease (lymphogranuloma)	59	51	46	38	22
Lymphosarcoma	17	13	11	5	5
Reticulum cell sarcoma	7	4	2	1	1
Multiple myeloma and plasmocytoma	8	6	3	2	—
Giant follicular lymphosarcoma	9	7	5	2	2
Reticulosis	1	1	1	1	1

Results were favorable in previously untreated cases, and dosages of 700 to 800 mg. were sufficient to produce improvement. When patients had undergone previous X-ray treatment, larger doses were required. Simultaneous radiotherapy and BCM treatment, with adequate precautionary measures, are feasible.

As to localization, all cases seem to react the same way. Peripheral, mediastinal, and abdominal glands can be treated equally well. In cases of spleno- and hepatomegaly, however, the danger of prolonged leukopenia demands greater caution in dosage.

In most cases the nodes are softer after the third injection. The fourth and fifth injections are followed by malaise and pyrexia. In the meantime the nodes become smaller, so that after the eighth and ninth doses of 100 mg. they gradually disappear completely. Peripheral nodes subjected to previous X-ray treatment do not change their size to any great extent. There are various exceptions, of course, to these generalizations. The fall in fever is usually followed by increase in appetite and by weight gain, and the sedimentation rate becomes normal.

Case history No. 5. K. J., a 30-year-old woman, had enlarged cervical and axillary nodes, and fever to 39° C. She had no history of previous treatment. After the third injection of BCM, her temperature dropped, her general condition improved, and the swollen glands became discrete and soft. The day following the sixth injection, pyrexia recurred for 2 days, during which period the nodes disappeared. Malaise and nausea cleared after the ninth injection. Since then the patient has had no complaints and no evidence of disease.

It is undeniable that the disease recurs in most cases. Still, BCM should be considered an advance as compared with nitrogen mustard because of the former's lower toxicity.

Lymphosarcoma

Our experiments with lymphosarcoma produced results similar to those obtained in chronic lymphocytic leukemia, but generally they were not as good. Some patients, however, remained asymptomatic for more than 2 years (5 of 17 cases). The favorable responses have been prolonged, and improvement has been evident after administration of a small amount of BCM.

Case history No. 6. Sz. S., a 46-year-old man, had an egg-sized cervical tumor that receded after 3 × 100 mg. BCM. To date, he has been asymptomatic for 2 years and is able to work full time.

The response seems to be "all-or-nothing," and in some cases the disease is completely refractory.

Case history No. 7. G. E., a 54-year-old female patient, was completely unresponsive to 600 mg. BCM. Although she developed a leukopenia of 1200, her large hard tumors were unchanged. It was necessary to discontinue treatment, and she died 2 months later.

Some cases resistant to BCM were also refractory to X-ray treatment. This may indicate that BCM, to some degree, resembles X-ray treatment in its effectiveness on the disease.

Reticulum Cell Sarcomas

No outstanding results were obtained in the reticulum cell sarcomas. Most of our cases proved to be resistant, and only a few (2 of 7) remained symptomless for a period longer than 6 months.

Case history No. 8. G. P., a 43-year-old driver, received 500 mg. BCM. After the treatment no evidence of the disease remained. He has continued to do heavy manual work for the past 2 years.

In treating reticulum cell sarcoma, larger amounts of the drug were required; the total dose in each case ranged between 1000 and 1200 mg. In most cases relapse occurred within 2 to 3 months.

Plasmacytomas and Multiple Myeloma

BCM alone resulted in an asymptomatic period only if given in very large doses.

Case history No. 9. Z. O., a female patient, received a total dose of 2000 mg. BCM given in 20 divided doses. She tolerated this amount well and remained asymptomatic for 4 months.

Much better results were obtained, however, by administering BCM in combination with urethan. To render the patient symptomless for approximately 12 months a daily dose of only 1 gm. (instead of the usual 3 to 4 gm.) urethan combined with a smaller amount of BCM was sufficient. After the sixth to eighth injection the elevated sedimentation rate fell, the appetite of the patient improved, and pain was diminished. The serum proteins did not return to normal until the fourth week, and bone roentgenograms were more delayed in showing improvement. The duration of benefit exceeded 12 months in only 2 patients, and these patients were asymptomatic for 14 months.

Brill-Symmers Disease

All 9 cases shown in the tables were taken from the practice of Szentklárai, who has made a special study of this disease. Szentklárai found that this condition responded well to BCM treatment; one case showed only a partial response.

Reticulosis

One case (benign) reacted favorably to treatment with BCM.

In summary, BCM represents an advance in the treatment of lymphomas. The best results were seen in lymphosarcoma and the second best ones in cases of reticulum cell sarcoma. We have had equally good results in Hodgkin's disease, with less toxicity than that produced by nitrogen mustard. The combination of urethan and BCM may be effective in multiple myeloma.

SARCOMAS

An evaluation of the cases treated is given in TABLE 5. Under the heading "subjective improvement" we have included cases in which the patient's appetite increased, his pain decreased, and his general condition improved, but in which no objective evidence of tumor regression could be demonstrated. Measurable regression of disease is listed under "objective improvement." Only 1 patient showed objective improvement.

TABLE 5

Diagnosis	Number of cases treated	No response	Improvement	
			Subjective	Objective
Pulmonary sarcoma	4	3	1	—
Osteogenic sarcoma	2	1	1	—
Fibrosarcoma	1	—	—	1

Case history No. 10. B. G., a child of 16 months, had a recurrent fibrosarcoma originating in the left cheek and covering the whole left eye. BCM reduced the tumor to half of its original size. The child continues under treatment. His appetite and his general condition are greatly improved, and normal development continues.

CARCINOMAS

Cancers of the Digestive Organs

The results obtained are shown in TABLE 6. Only 1 patient showed objective improvement.

TABLE 6

Diagnosis primary site of carcinoma	Number of cases treated	No response	Improvement	
			Subjective	Objective
Esophagus	1	—	1	—
Stomach	13	12	1	—
Intestine	3	2	—	1
Pancreas	3	3	—	—
Gall bladder	1	1	—	—
Rectum	7	5	2	—
Total	28	23	4	1

Case history No. 11. S. K. had an inoperable carcinoma of the intestine, and a colostomy was performed. The patient received 1000 mg. BCM. When an attempt was made to close the colostomy 1 year later, the tumor appeared to have regressed slightly.

The other cases, notwithstanding some slight subjective improvement, showed the usual picture of progression of the disease.

Cancers of the Respiratory Tract

The results obtained are shown in TABLE 7.

Case history No. 12. E. M., a 63-year-old female, had a proved anaplastic carcinoma of the lung. Combined treatment with BCM and radiotherapy produced a prolonged period of symptomatic improvement. She did not require further thoracenteses, and roentgenograms of the chest showed no abnormalities. She has had no complaints for more than 1 year, and has gained 8 kg.

In some other cases BCM appeared to inhibit the progression of the disease, to decrease pain, and to improve the general condition of the patient. In 6 patients, proved at thoracotomy to be inoperable, it was possible to prolong life for a year.

TABLE 7

Diagnosis primary site of carcinoma	Number of cases treated	No response	Improvement	
			Subjective	Objective
Larynx	2	1	—	1
Lung	57	50	6	1
Malignant tumor of mediastinum	4	3	1	1
Total	63	54	7	3

In summary, we believe that in neoplasms of the lung, when radiotherapy and surgical intervention are not feasible, BCM should be tried. We are now trying to develop methods of inhibiting the progression of the disease by using various combinations of drugs and radiation.

Endocrine Tumors

Results in carcinomas originating in various glands are summarized in TABLE 8. In patients with carcinomas of the breast and of the ovary, only subjective improvement was obtained. Hormone therapy may be much more effective. One patient with carcinoma of the cervix showed a favorable response.

TABLE 8

Diagnosis primary site of carcinoma	Number of cases treated	No response	Improvement	
			Subjective	Objective
Breast	13	8	5	—
Ovary	8	6	2	—
Uterine cervix	5	4	—	1
Thyroid	1	1	—	—
Prostate	1	1	—	—
Total	28	20	7	1

Case history No. 13. W. I., a 61-year-old female, had had a hysterectomy for carcinoma of the cervix 3 years ago. She relapsed 1 year later with pulmonary metastases. The process was arrested after treatment with 1000 mg. BCM. At present, 2 years later, her condition is good and she is able to do her housework.

Miscellaneous Neoplasms

The results of treatment with BCM are summarized in TABLE 9.

Case history No. 14. Sz. J., a 52-year-old woman, had hepatic metastases, proved by laparotomy 2 years earlier, after removal of a parotid tumor. After she received 1500 mg. BCM her liver decreased in size. Fourteen months after treatment the liver has not changed in size, and the patient is attending an outpatient clinic.

TABLE 9

Diagnosis primary site of cancer	Number of cases treated	No response	Improvement	
			Subjective	Objective
Seminoma testis...	3	2	1	
Hypernephroma.....	1	1		
Malignant melanoma..	2	2		—
Retinoblastoma*.....	1	—	—	
Glomus tumor*....	1			
Meristoma*..........	1	-	—	
Basal cell carcinoma.	6	-	5	1
Tonsil...............	2	2		
Metastases, primary undiagnosed......	9	9		
Pleomorphic adenocarcinoma of parotid gland............................	2	1	–	1
Total..	28	17	6	2

* Under treatment.

In all other cases, treatment with BCM was completely unsuccessful and the course of disease was unchanged.

In a total of 154 patients with various types of cancer treated with BCM, 119 showed no response, 26 obtained some subjective improvement, and in 9 (6 per cent) there was objective improvement. The number showing subjective improvement could not be regarded as significant, since BCM caused indisposition in at least as many cases.

SIDE EFFECTS

Nausea and Vomiting

BCM may cause sickness, malaise, nausea or, in the more sensitive cases, vomiting. These symptoms, while reminiscent of those caused by nitrogen mustard, are not as severe. Such reactions occur in approximately 45 per cent of the cases. They depend on the amount of the drug injected, previous treatment, and individual sensitivity. Doses less than 1 mg./kg. do not cause a reaction, but it occurs regularly when the dose is over 3 mg./kg. Patients who received previous cytostatic or X-ray therapy are more sensitive. On the other hand, repeated treatments with BCM do not increase sensitivity.

Nausea and malaise usually last about 18 to 36 hours. Consequently, the patient is restored to his usual state before the second dose, which is administered 48 hours later. It should be noted that patients who are unduly sensitive at the beginning of treatment become more so later, so that a dose of 0.2 mg. kg. (10 to 20 mg.) is enough to cause a reaction. This immediate reaction to BCM may be due to two factors: first, the pharmacological action of the drug and, second, the breakdown of cells due to the cytotoxic action of the drug. The former component of the reaction may be relieved by sedatives and vitamin B_6, but the "cytostatic shock" does not respond to any treatment. Patients are willing to tolerate these effects, however, in view of the favorable results of treatment. Szentklárai, who dissolved BCM in 100 mg. vitamin C, has observed less severe side effects.

Bone Marrow Depression

The most careful control of dosage of BCM is needed in cases of chronic lymphocytic leukemia, acute leukemia, and lymphosarcoma, where even very small doses are apt to produce severe hematological effects. Special attention is given to patients with a history of previous treatment.

Patients with lymphomas and leukemia tolerate smaller doses of BCM than patients with carcinoma. Among 154 cancer patients only a single case with agranulocytosis occurred, as compared to 7 of 164 cases of lymphomas and leukemia. Moreover, in cases of carcinoma large amounts were given.

The earliest sign of agranulocytosis due to BCM is the disappearance of the myeloblasts and promyelocytes from the bone marrow. Later, the number of the more mature cells decreases and the remaining ones degenerate. These findings precede the decrease of the peripheral cells. All lymphoid elements disappear. The megakaryocytes also decrease, followed by the erythroblasts. Only single plasma cells or big lymphoid reticulum cells may be found microscopically, and finally the marrow disappears macroscopically too. We had only a single case where regeneration occurred, and in this case the restitution of erythropoiesis was the first step toward regeneration.

Case history No. 15. Sch. L., a 54-year-old female with Hodgkin's sarcoma and high fever was treated with BCM. She received a total of 900 mg., 1.5 mg./kg. on alternate days. After an afebrile period of 6 weeks, fever returned, and she received 600 mg. BCM. The WBC dropped to 2000 and treatment was discontinued. ACTH did not alter the situation. Sternal aspiration showed an empty marrow. She died 8 days later. On the day of death WBC was 800, RBC 1,500,000, thrombocytes 50,000, in spite of transfusions of 600 to 800 cc. of blood daily. In addition, this patient had received Mitomen (2 × 25 mg.).

Case history No. 16. H. E., a 23-year-old female, received X-ray therapy to multiple parts and actinomycin C (3.0 mg.) 2 years previously. She received three courses of BCM, to a total dose of 2800 mg. No side effects occurred. Three months after the last treatment, anemia and then leukopenia developed, with a leukocyte count of 2000 and an erythrocyte count of 1,000,000. Platelets were normal, and there was no bleeding. After treatment with blood transfusions and ACTH, bone marrow regeneration was rapid.

In summary, the danger of agranulocytosis from BCM is practically nonexistent in cases of cancer. A more cautious course is indicated in cases of leukemia and lymphomas, especially when the patient has been treated with other cytostatic agents.

In two cases reactivation of an old tuberculous process as a result of BCM treatment has been observed. In each case the process later responded favorably to appropriate treatment.

A possible hepatotoxic action is noted in the following case.

Case history No. 17. T. O., a 59-year-old man with carcinoma of the lung, received 1100 mg. BCM. The disease was arrested, but 5 weeks later icterus occurred and liver function tests were slightly positive. The patient improved on an adequate diet and ACTH. BCM administration was then renewed, and icterus returned 8 hours after injection of 50 mg. BCM. When a similar episode occurred 4 months later, administration of BCM was discontinued.

Similar effects were observed in two other cases.

Dosage Problems

BCM is given intravenously. Pleural or peritoneal administration has no special advantage, and no extraordinary effects can be expected from intratumoral injections.

A number of observations were made concerning dosage. The longer the period between the injections the less effective they were. Administration every 24 hours was very effective, but the danger of side effects was great. According to our experience the best course is to give BCM every 48 hours.

It is difficult to answer the question: how long should BCM be administered? The duration of treatment will vary with the type of case, the evidence of a favorable effect, and the desired response. Inadequate treatment may soon be followed by relapse, and if one BCM treatment follows too closely on another, the bone marrow injury may be increased and it may be necessary to reduce the dosage so that it is no longer adequate.

For lymphomas and leukemias we recommend that a patient receive one adequate course of treatment, even if a protracted treatment period is necessary, rather than numerous treatments of short duration. Treatment cannot be repeated within 3 months with any hope of success even if otherwise indicated. Repetition of treatment after a 6-month interval is not dangerous.

The picture is more complicated for carcinomas. Most of the patients do not respond to the drug; moreover, there are no side effects of treatment. In these cases an increase in the single dose and total dose is justified.

According to one technique[25] the patient receives a combination of chlorpromazine (Largactil), promethazine (Phenergan), and meperdine (Dolantin), after which he tolerates as much BCM as 10 mg./kg. In a second method, larger doses are given more frequently and the treatment period is prolonged.

Cumulation

How long does BCM exert its effect after injection? The metabolism of BCM in the body is not known. Its effects on the marrow persist long after the treatment is completed. Examination of the bone marrow shows that the effects of BCM may continue for approximately 6 to 8 weeks. A prolonged period of bone marrow depression may occur while the disease remains static and, in some cases, bone marrow recovery proceeds during a continuing remission of the disease.

Case history No. 18. B. I., a 42-year-old man, diagnosed as having Hodgkin's disease, had been treated with nitrogen mustard. He was admitted with enlarged mediastinal nodes that were causing obstructive respiratory symptoms. Despite a low leukocyte count he received 100-mg. doses of BCM, to a total of 1200 mg., under protection with ACTH and blood transfusions. Hemorrhagic manifestations appeared, but the lymph nodes disappeared. The patient recovered from the thrombocytopenia and leukopenia, and he was asymptomatic for 8 months. Then he spontaneously developed evidence of a myelophthisis. In the meantime his disease remained quiescent.

COMBINATION WITH OTHER THERAPY

In cases where BCM itself is inadequate, we are attempting to enhance its effects by combining it with other agents. The following combinations are under study: (1) BCM and Mitomen; (2) BCM and sarcolysin; (3) BCM, Mitomen, and sarcolysin; (4) BCM and radiotherapy; (5) BCM and hormonal treatment; (6) BCM and urethan; (7) BCM and stilbamidine; (8) BCM and Myleran. We do not combine it with triethylene melamine or with nitrogen mustard, since these agents also have a depressant action on the bone marrow.

Finally, in cooperation with several other institutions we have started a trial of BCM as a prophylactic treatment on postoperative and preoperative cases. The laboratory studies have indicated that this may be a promising procedure.[18-20]

The results of recent studies done with BCM have been published in various European journals.[24-27]

Conclusions

The therapeutic value of 1,6-bis(β-chloroethylamino)-1,6-deoxy-D-mannitol dihydrochloride (BCM, Degranol) has been explored in 318 patients with neoplastic disease. (1) BCM proved to be very effective in cases of lymphomas and leukemias. (2) In cases of malignant diseases, favorable results were limited to single cases, but it is believed that many other therapeutic possibilities should be studied. (3) The lymphomas and leukemias require smaller total amounts of BCM than the carcinomas. (4) Among the side effects, bone marrow depression is the most serious. Other side effects that may accompany administration of BCM are malaise, nausea, and vomiting. The management of these undesirable effects is discussed. (5) Current studies on the combination of BCM with other agents are noted.

References

1. Sellei, C. & J. Jány. 1935. Biochem. Z. **275**: 234.
2. Gilman, A. & F. S. Philips. 1946. Science. **103**: 409.
3. Rhoads, C. P. 1946. J. Am. Med. Assoc. **131**: 656.
4. Karnofsky, D. A. 1948. New Engl. J. Med. **239**: 260, 299.
5. Haddow, A., R. J. C. Harris, G. A. R. Kon & E. M. F. Roe. 1948. Trans. Roy. Soc. London. **A 241**: 147.
6. Matthews, W. B. 1950. Lancet. **1**: 896.
7. Everett, J. L., J. J. Roberts & W. C. J. Ross. 1953. J. Chem. Soc. : 2386.
8. Galton, D. A. G., L. G. Israels, J. D. N. Nabarro & M. Till. 1955. Brit. Med. J. **2**: 1172.
9. Larionov, L. F., A. S. Khokhlov, E. N. Shkodinskaja, O. S. Vasina, V. I. Troosheikina & M. A. Novikova. 1955. Lancet. **2**: 169.
10. Bergel, F. & J. A. Stock. 1954. J. Chem. Soc. : 2409.
11. Druckrey, H. 1954. Deut. med. Wochschr. **79**: 1667.
12. Ishidate, M. 1951. Proc. Japan Acad. **27**: 493.
13. Sellei, C. & S. Eckhardt. In press.
14. Masszi, F., L. Németh, C. Sellei & G. Till. 1952. Kisérletes Orvostudomàny. **4**: 248.
15. Vargha, L. 1955. Naturwissenschaften. **42**: 582.
16. Dumbovich, B. 1956. Magyar Kém. Lapja. **11**: 34.
17. Vargha, L., L. Toldy, Ö. Feher & S. Lendvai. 1957. J. Chem. Soc. : 805.
18. Kellner, B., L. Németh & C. Sellei. 1955. Naturwissenschaften. **42**: 582.
19. Kellner, B. & L. Németh. 1956. Z. Krebsforsch. **61**: 165.
20. Lapis, K. & L. Németh. 1956. Klin. Wochschr. **34**: 864.
21. Kellner, B. 1957. Acta Unio Intern. contra Cancrum.
22. Szentklárai, F. Unpublished data.
23. Sellei, C., S. Eckhardt & F. Hartai. 1957. Medizinische. **19**: 722.
24. Sellei, C., S. Eckhardt, F. Hartai & B. Dumbovich. 1956. Lancet. **1**: 785.
25. Sellei, C., S. Eckhardt, F. Hartai & E. Molnár. 1956. Orvosi Hetilap. **97**: 999.
26. Kellner, B. & L. Németh. 1956. Orvosi Hetilap. **97**: 991.
27. Sellei, C. & Z. Antolóczy. 1956. Wien. Z. inn. Med. u. Grenzg. **37**: 337.

PALLIATION OF CARCINOMATOSIS WITH ASCITES BY MEANS OF HEMISULFUR MUSTARD

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Hemisulfur mustard (2-chloro-2′-hydroxydiethyl sulfide, HSM) was prepared for pharmacological and clinical study in the course of an attempt to develop derivatives of this mustard that would be susceptible to enzymatic hydrolysis. The rationale of such an approach was to develop less toxic derivatives of the mustards, such as a phosphoric acid ester of hemisulfur mustard, from which a reactive mustard moiety (HSM) might be liberated within the cancer cell particularly rich in the enzyme phosphatase. Such a derivative has not yet been prepared in a form sufficiently pure for study. While synthetic work on the ester was in progress, the toxicity of HSM itself was first cautiously explored in a patient with extensive metastases from a carcinoma of the prostate refractory to all known therapeutic measures who appeared to be in the terminal stages of his disease. Following administration of HSM, a remission of symptoms for about eight months resulted. Even though the remission may have been unrelated to the administration of HSM, and since nitrogen mustards are of little value in carcinoma as compared to lymphoma, this observation in a case of epithelial tumor was sufficiently significant to warrant further study.

Pilot survey studies in humans with a variety of carcinomas revealed that in some patients carcinomatosis, particularly of ovarian origin and with ascites, was palliated. When palliation was observed, it generally consisted of suppression of formation of ascitic fluid as well as decrease in size of tumor masses when present, decrease in pain, and increase in appetite, strength, and ability to ambulate.

In the first group of 31 patients reported,[1] patients appeared to be temporarily benefited by HSM. The longest remission was 10 months, and the shortest was several weeks. In the second group of 30 patients reported,[2] 14 were temporarily benefited. The longest remission of 29 months was observed in a patient with a mixed mesodermal tumor of the endometrium. The toxic reactions consisted of nausea, vomiting, malaise, and tremor. Bone marrow depression was not observed.

Hemisulfur mustard is now available commercially* and is supplied in solution in dry ether in sealed glass ampoules, which are stored at −10 to −20° C. The ether-mustard solution (100 mg. per cc.) is diluted with alcohol (4 cc. per cc. of ether) and then mixed with saline immediately before injection at the bedside. Care should be taken to obtain a colloidal solution so as not to inject ether solution neat. Injection is made through polyethylene tubing inserted into the brachial vein as far as the superior vena cava. This is necessary to avoid thrombophlebitis. The initial dose is 100 to 200 mg. and the total dose given in 3 to 4 injections is 300 to 900 mg.

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* Available in ampoules from Dajac Laboratories, Chemical Division, The Borden Company, Philadelphia, Pa.

Further clinical study with HSM appears warranted. A definitive clinical trial in cases of carcinomatosis with ascites would also probably be worthwhile. The major significance of these observations to us, however, is in the lead for developing chemotherapeutic agents that may prove to be better than the current agents for carcinoma.

References

1. Seligman, A. M., A. M. Rutenburg, L. Persky & O. M. Friedman. 1952. Effect of 2-chloro-2′-hydroxydiethyl sulfide (hemisulfur mustard) on carcinomatosis with ascites. Cancer. **5**: 354.
2. Rutenburg, A. M. & A. M. Seligman. 1956. Palliation of carcinomatosis with ascites by means of hemisulfur mustard (2-chloro-2′-hydroxydiethyl sulfide). New Engl. J. Med. **255**: 361.

OXAPENTAMETHYLENEDIETHYLENE THIOPHOSPHORAMIDE (OPSPA)* IN THE TREATMENT OF HUMAN CANCER†

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N-(3-Oxapentamethylene)-*N*′,*N*″-diethylene thiophosphoramide (OPSPA) has shown a significant tumor-inhibitory effect on transplanted tumors in animals.[1] The purpose of the study reported in this communication was to learn something of the toxic manifestations and therapeutic effectiveness of OPSPA in patients with a wide variety of malignant lesions. The metabolic fate of OPSPA in animals[2, 3] and in humans[4] has been reported previously.

Consideration of this report can be accomplished most logically by review of two separate phases of the research program. The first will be referred to as "OPSPA by Persistent Therapy" (Group I) and the second as "OPSPA by Intermittent Courses" (Group II). In both phases of this work emphasis was placed on the selection of patients with solid tumors not suited to curative therapy by conventional measures.

Group I. OPSPA by Persistent Therapy

Cases treated. TABLE 1 illustrates the wide variety of malignant neoplasms selected for study. Among the 102 cases included in this table are 18 patients treated curatively by surgery (that is, no known residual tumor after surgery), but known to present a poor prognosis because of the nature of their disease. These cases, which were treated prophylactically, are too few in number for statistical evaluation, but the rapid and fatal termination of 11 of them due to progressive disease may be worthy of note. The 18 cases described as examples of prophylactic therapy included 9 gastrointestinal carcinomas, 6 glioblastomas, 2 sarcomas, and 1 thyroid carcinoma.

Methods. OPSPA was given chiefly intramuscularly, subcutaneously, or intratumorally. Less commonly the material was given intravenously, intra-arterially, or by the intracavitary route. The oral route was not used because of previous demonstration of ineffectiveness orally in animals.[1] Saline, corn oil, or carbowax was used as a vehicle for the drug at different times or in different patients, and the material was prepared at a concentration of 10 or 20 mg. of drug per milliliter of vehicle, usually the former.

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* The OPSPA used in the first portion of the study reported here was generously supplied by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y., through J. M. Ruegsegger, Medical Director. OPSPA to complete the study was prepared by Charles Heidelberger and P. M. Bhargava, of the McArdle Laboratories, University of Wisconsin, Madison, Wis.

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‡ Clinical Fellow, Cancer Research, American Cancer Society.

TABLE 1
GROUP I. OPSPA BY PERSISTENT THERAPY
Diagnosis and Result of Therapy

Diagnosis	Number of cases	Local tumor effect	General tumor effect
Carcinoma of gastrointestinal tract	26	0	0
Sarcoma	14	0	0
Squamous cell carcinoma of head and neck	11	0	0
Glioblastoma multiforme	9	0	0
Carcinoma of breast	8	2	0
Melanoma	7	1	0
Carcinoma of lung	6	0	0
Lymphoma	6	2	1
Hypernephroma	3	0	0
Multiple myeloma	2	0	1
Carcinoma of ovary	2	1	0
Carcinoma of pancreas	2	0	0
Leukemia	2	0	2
Carcinoma, primary site unknown	2	0	0
Carcinoma of thyroid	1	0	0
Carcinoma of cervix	1	0	0
Total	102	6	4

In the early phase of this study there was no previous human experience with OPSPA upon which to rely and, as a result, no strict dosage schedule was followed, and the protocol was changed as experience was gained. In summary, there were two common practices. One was to administer a large intratumoral injection initially and to follow this with long-term therapy at weekly, or more frequent, intervals. This plan was followed most regularly when an inoperable lesion was discovered at exploratory surgery. A second procedure evolved: giving 0.75 mg. per kg. on day 1 and 0.4 mg. per kg. on days 3 and 5. Thereafter, 5 mg. or 10 mg. was given once or twice weekly, as tolerated, for weeks or months. The white blood count (WBC) was used as the most practical measure of tolerance.

Toxic manifestations. Tissue reaction at the site of injection of OPSPA was not encountered. Gastrointestinal disturbance was virtually nonexistent. Evidence of renal damage was not detected. In fact, the only evidence of toxicity was a pancytopenia that tended to appear first 10 to 14 days after initiation of therapy and that became severe if very large dosages were given or if small doses were continued for many weeks. A summary of the most severe reactions, with dosages employed, is listed in TABLE 2. Other patients showed profound pancytopenia with occasional bleeding from mucous membranes, prolonged bleeding after biopsy, and cutaneous ecchymoses, but all recovered.

Results. The responses to therapy seen in Group I are recorded in TABLE 1. Objective evidence alone is considered, and such a record justly fails to pic-

TABLE 2
GROUP I. OPSPA BY PERSISTENT THERAPY
Toxic Effects

Sex, age, diagnosis	Dosage and duration of therapy	Result. WBC in thousands per cubic millimeter
1. Male, 59, squamous cell carcinoma, lung	600 mg. in 11 days	Pancytopenia (WBC 0.3) Patient expired on 24th day. Autopsy performed.
2. Male, 70, squamous cell carcinoma, pharynx	380 mg. in 43 days	Pancytopenia (WBC 0.1) Patient expired on 49th day. Autopsy performed.
3. Female, 39, reticulum cell sarcoma, mediastinum	260 mg. in single injection	Pancytopenia (WBC 0.15) Patient expired on 16th day. Autopsy performed.
4. Male, 56, anaplastic carcinoma, lung	200 mg. in single injection	Pancytopenia (WBC 0.3 on 11th day, patient recovered and WBC reached 6.8 on 26th day). Patient expired later of cancer.

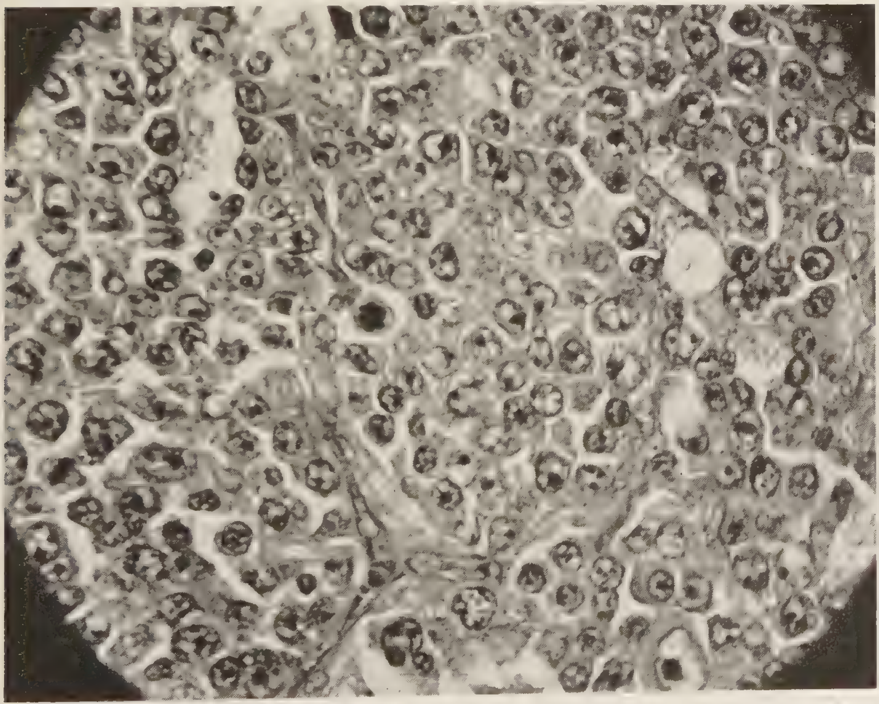

FIGURE 1. O. G. Slide No. 56-3288. Reticulum cell sarcoma. Biopsy from chest wall before treatment. Note sheets of cells with large nucleoli and two mitotic figures. × 475.

ture the numerous occasions when patients were totally convinced and investigators were tempted to believe that a favorable response had occurred. Six patients showed tumor regression at sites of injection of the drug. Four patients showed regression of neoplastic disease of more than local extent. These 4 cases will be reviewed briefly.

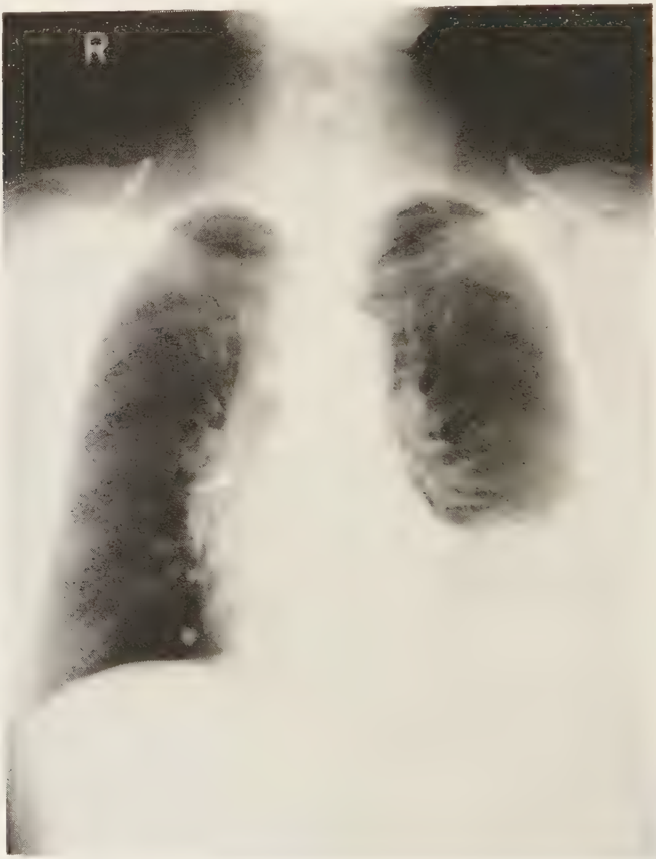

FIGURE 2. O. G. X-ray film, April 12, 1954. Note pleural involvement partially obscuring left lower lobe.

Case T. K. A 68-year-old male with widespread multiple myeloma diagnosed histologically. Upon admission the patient was disoriented, the eyelids were swollen shut by edema and by the pressure of a neoplastic mass at the left orbital margin. OPSPA was given I.M. to a dosage of 130 mg. in the first 20 days and 2 times weekly thereafter, as tolerated. The facial mass decreased markedly, and simultaneously the edema and mental confusion cleared. This patient was improved for approximately 3 months and then gradually succumbed to progressive disease in spite of continued therapy.

Case O. G. A 67-year-old female with reticulum cell sarcoma extending through the left chest wall and involving the left pleural space (FIGURES 1 and 2). On April 20, 1954, OPSPA was injected about the biopsy site below the left breast, and 90 mg. was given over a 5-day period. Thereafter small injections were given by the patient's referring physician 2 or 3 times weekly as tolerated. One month later the lesion at the site of biopsy had healed and the intrathoracic changes had cleared. A chest X ray taken on April 3, 1957 (FIGURE 3), shows that pulmonary or pleural involvement has not recurred. The patient showed no evidence of neoplastic disease at that time (36 months).

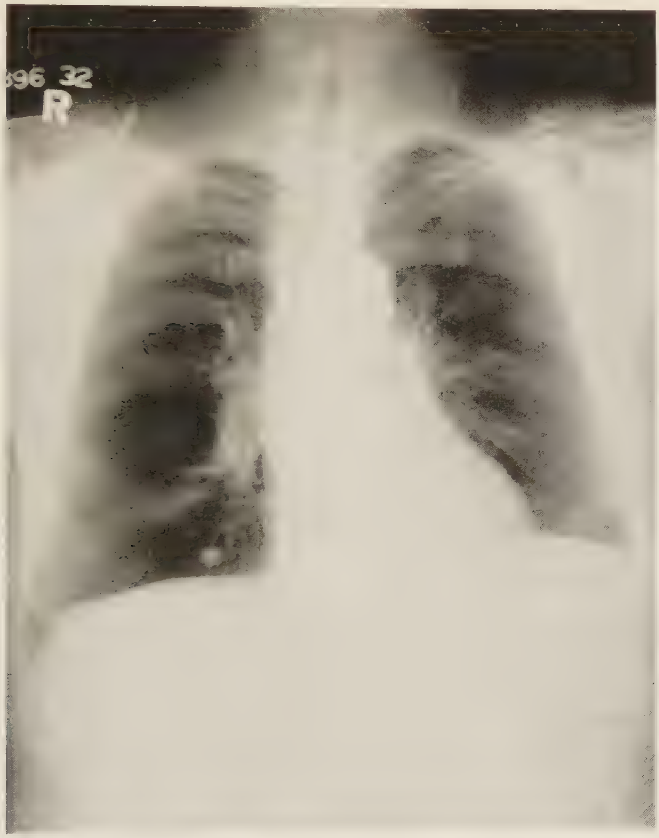

FIGURE 3. O. G. X-ray film, January 2, 1957. Note improvement in the lower portion of the left hemithorax. This film was substituted for a similar view on April 3, 1957 because of its superior technical quality.

Case R. H. A 49-year-old male with a diagnosis of chronic myelogenous leukemia. At the start of therapy the WBC was 159,500, the liver was enlarged, and the spleen was palpable 15 cm. below the left costal margin. OPSPA was given I.M., and the patient received 290 mg. from November

24, 1954, to March 3, 1955. During this time the patient had gained in weight, the Hb had increased, the WBC dropped to 5000, and the liver and spleen became impalpable. Supportive transfusions were given during the summer of 1955, but the patient did well until after May, 1956, when anemia became a problem and the white count increased. The patient died on September 16, 1956.

Case J. L. A 61-year-old female with diagnosis of chronic lymphocytic leukemia based on bone marrow biopsy (FIGURE 4). Findings before therapy included: WBC 32,550, Hb 10.0, liver 8 cm. below the right costal margin, and spleen 10 to 15 cm. below the left costal margin. OPSPA was given I.M., 10 mg. daily on 12 consecutive days, starting January 19, 1955. One month later the patient was markedly improved, and on April 7, 1955, the following findings were recorded: WBC 5050, Hb 12.8, liver not palpable, and spleen 2 to 3 cm. below the left costal margin on deep inspiration. The patient had gained 21 pounds. On February 7, 1957 (24 months) the patient felt well, Hb and WBC were normal, and the liver and spleen were not palpable.

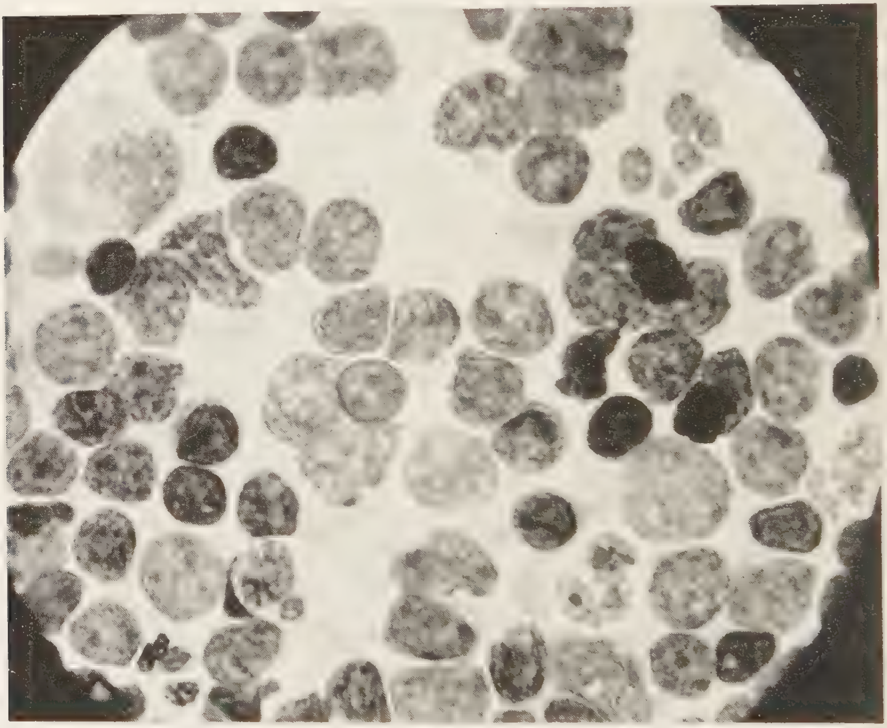

FIGURE 4. J. L. Slide No. 55-41. Chronic lymphocytic leukemia. The identifiable cells include 5 of the granulocytic series, 6 mature lymphocytes, and 1 lymphoblast; the remainder are young lymphocytes. × 1450.

Group II. OPSPA by Intermittent Courses

Cases treated. TABLE 3 presents a list, by diagnosis, of patients treated in this phase of the work. Selection of cases, as in Group I, was limited to patients who could not expect curative management by conventional therapy. Sixty-eight patients were given 93 courses of treatment. In this group, also, emphasis was placed on selection of solid tumors, and all diagnoses were established by histology or cytology (3 cases).

TABLE 3
GROUP II. OPSPA BY INTERMITTENT COURSES
Diagnosis and Result of Therapy

Diagnosis	Number of cases	Number of courses	Local effect	Distant effect
Carcinoma of breast	11	16	1	1
Melanoma	8	9	0	0
Carcinoma of gastrointestinal tract	8	8	1	0
Carcinoma of ovary	7	12	1	1
Carcinoma of lung	6	9	0	0
Hypernephroma	5	8	0	0
Sarcoma	5	7	0	0
Abdominal carcinoma, primary site uncertain	5	7	0	0
Squamous cell carcinoma of head and neck	4	6	0	0
Lymphoma and myeloma	4	5	0	3
Carcinoma of genital system	2	2	0	0
Carcinoma of uterine cervix, uterine corpus, and Wilms's tumor (1 each)	3	4	0	0
Total	68	93	3	5

Methods. In this series OPSPA was given in one or more courses of 5 consecutive days' treatment. When possible, courses were repeated after intervals of 4 to 6 weeks. As a general rule the medication was given intravenously (69 courses), but 12 courses were given by the intracavitary route when effusion was present, 11 courses were given intramuscularly, and 1 patient who had received the first course of therapy intravenously was given a second course by intratumoral injection. Because of the reported favorable results obtained by combining nitrogen mustard with adrenocorticotrophic hormone (ACTH) or cortisone,[5] one of these hormones was given in conjunction with OPSPA to 35 patients in 49 courses (TABLE 4). When ACTH was used (21 patients, 31 courses) it was started 1 or 2 days before therapy with OPSPA at a dosage of 60 mg. every 6 hours and was discontinued 1 or 2 days after completion of the 5-day course of OPSPA therapy. When cortisone was used (14 patients, 18 courses) it was started a day or two before OPSPA therapy at a dosage of 50 mg. every 4 hours. This dose was continued for 8 or 9 days, and the patients were discharged to continue cortisone at 25 mg. 4 times daily between courses. Six patients who received hormone therapy were also given triethylene melamine (TEM), receiving the calculated dosage of OPSPA on days 1, 3, and 5 of therapy and 2.5 mg. TEM on days 2, 4, and 6. This plan was followed in 6 courses and, again, the procedure was based on the report cited above.[5] Thirty-two patients were treated as a base-line group with OPSPA alone. These 32 patients received a total of 37 courses of therapy. Four patients who received ACTH or cortisone were also given courses of OPSPA alone.

TABLE 4
GROUP II. OPSPA BY INTERMITTENT COURSES
Method of Treatment and Result

Drug	Number of cases	Number of courses	OPSPA 0.4 mg./kg./day × 5 or more. Number of courses	WBC to 3500 or less. Number of courses
OPSPA alone	32	37	34	7
OPSPA + TEM	1	1	0	1
OPSPA + ACTH	21	31	14	6
OPSPA + cortisone	14	18	13	5
OPSPA + hormone + TEM	6	6	0	1
Total	74*	93	61	20

* This total is not the same as the number of cases treated because, on 6 occasions, patients received more than one method of therapy.

The dosage of OPSPA employed started at 0.3 mg./kg./day × 5, and was gradually increased in subsequent patients or courses of treatment until the dosage of 0.5 mg./kg./day × 5 was selected as a maximum initial safe dose. Forty-nine patients received 61 courses of therapy at a dosage of 0.4 mg./kg./day × 5 or more (TABLE 4). One patient received 0.85 mg./kg./day × 5. Two received 0.6 mg./kg./day × 5.

Toxic manifestations. In this series, no toxic effects were encountered that had not been experienced in the Group I cases. Depression of peripheral white blood counts to 3500 cells per cubic millimeter or less occurred (TABLE 4) in 20 of 93 courses (22 per cent). Two of these examples, however, were in patients who at the beginning of therapy had counts below 3.5 thousand, and 2 were in patients who also received 7.5 mg. of TEM. The remaining 16 cases (17 per cent) with significant hematopoietic depression occurred in 6 patients who received OPSPA alone, in 6 patients who received OPSPA plus ACTH, and in 4 patients who received OPSPA plus cortisone. The individual variation in response is illustrated by the fact that a total dosage of 2.5 mg./kg. of OPSPA in a 5-day course depressed the WBC to counts as low as 1800 cells, although the same regimen produced only minor hematopoietic depression in other cases. One patient began his second course of OPSPA with a WBC of 10,300. After receiving a total dose of 2.4 mg./kg.

in 5 days his lowest WBC was 2800. The same patient started a third course with a WBC of 21,300. On this occasion he received a total dose of 2.6 mg./kg. of OPSPA in 5 days and his lowest WBC was 6400. Characteristically, the lowest WBC occurred in the third or fourth week after initiation of therapy. In those cases with the most profound WBC depression this tended to occur earlier, frequently in the second or third week. The most severe examples of hematopoietic depression are outlined in TABLE 5.

TABLE 5
GROUP II. OPSPA BY INTERMITTENT COURSES
Toxic Effects

Case No., sex, age, diagnosis	Therapy and dosage	Result. WBC in thousands per cubic millimeter
No. 12. Female, 74, carcinoma of breast	OPSPA 0.85 mg. kg. × 5 plus cortisone (two previous courses)	Pancytopenia (WBC 2.1) Patient expired on 28th day. Referring physician states patient died cardiac death with no evidence of drug toxicity.
No. 30. Female, 59, squamous cell carcinoma of cervix	OPSPA 0.5 mg. kg. × 3 plus TEM 2.5 mg. × 3 (one previous course)	Pancytopenia (WBC 0.1) Patient expired on 25th day.
No. 72. Male, 38, giant follicular lymphoblastoma	OPSPA 0.5 mg. kg. × 5. Previous X-ray therapy. X-ray therapy started on 17th day after chemotherapy (WBC 3.6) because of rapidly progressive disease	Pancytopenia (WBC 0.35) Patient expired on 42nd day.

Bone marrow studies were done routinely before and after therapy until it was concluded that this was not a useful index of drug effect in patients with solid tumors. Evidence of bone marrow depression after therapy was seen in only 6 of 29 cases. It is of some interest to note that tumor cells were identified in the marrow specimens from 6 of 43 patients.

Platelet counts or estimations were also done routinely on all cases. As a rule platelet depression was proportional to the peripheral WBC. In every instance, when there was profound WBC depression there was also a marked decrease in platelets.

Results. Five of the 68 patients treated showed measurable influence of treatment upon lesions selected for evaluation before treatment. It should be noted that 3 of these cases received intravenous medication, but 2 cases were given intracavitary OPSPA for malignant effusion. Three cases showed effects that require special evaluation. All 8 cases (TABLE 3) will be reviewed briefly.

Case 7. Male, 26 yr., with a diagnosis of lymphoblastoma. Treatment consisted of 1.6 mg./kg. of OPSPA intravenously in 5 days plus ACTH during one course, and 2.7 mg./kg. of OPSPA plus ACTH during the second course. Following both courses large scalp masses subsided promptly, but recurred rapidly. There was definite evidence of tumor effect, but no patient benefit.

Case 12. Female, 74 yr., with a diagnosis of carcinoma of the breast. Treatment consisted of three 5-day courses of intravenous OPSPA at dosages of 1.4 mg./kg. during the first course, 2.6 mg./kg. during the second course, and 4.3 mg./kg. during the third course. ACTH was given with the first course of therapy and cortisone with the next two. There was almost complete epithelialization of a large malignant ulcer over the left breast area for a period of approximately 3 months. There was definite evidence of tumor effect and of patient benefit.

Case 25. Female, 71 yr., with a diagnosis of ovarian carcinomatosis with ascites. Treatment consisted of 4 courses of intraperitoneal OPSPA in doses ranging from 1.8 mg./kg. to 2.7 mg./kg. during each 5-day course. The patient was given ACTH with the first 3 courses and OPSPA alone with the last. During the 12 weeks before treatment an estimated 25,000 cc. of ascitic fluid was removed. During the 24 weeks after therapy 9500 cc. was removed and this was formed, chiefly, during the last few weeks. There was definite influence upon the rate of fluid formation and definite patient benefit.

Case 51. Female, 42 yr., with a diagnosis of carcinoma of the breast and pleural effusion. The patient received one course of intrapleural OPSPA at a dose of 2.0 mg./kg. During 4 weeks prior to therapy 9100 cc. of pleural fluid was removed, and during 6 weeks after therapy 1450 cc. was removed. There was definite, brief evidence of decreased pleural effusion but no significant benefit to the patient.

Case 65. Female, 63 yr., with a diagnosis of lymphosarcoma of the chest wall and pleura. This patient received a course of intravenous OPSPA at a dose of 2.5 mg./kg. There was a measurable decrease in the size of the chest-wall mass for a period of approximately 6 weeks, but the mass did not disappear. There was definite evidence of effect on the tumor, but no certain benefit to the patient.

The next three cases are presented separately for special consideration. The special feature of the first and second cases is that the dominant evidence was control of tumor growth and not regression. The special feature of the third case is that the 5-week interval between administration of Au^{198} and the use of OPSPA may cast doubt upon the validity of the evidence for OPSPA effect in spite of the clinical course to be described.

Case 29. Female, 54 yr., with a diagnosis of recurrent ovarian carcinoma. Treatment consisted of 3 courses of intravenous OPSPA at dosages of 2.1 mg./kg., 3.0 mg./kg., and 2.5 mg./kg. ACTH was given during the first course and cortisone with the next two. A pelvic mass had increased rapidly in size over a period of weeks before therapy, and the patient's activity had become curtailed. For a period of 24 weeks after treatment the pelvic mass and abdominal girth decreased slightly, possibly insignificantly, and the patient's functional capacity improved markedly. Thereafter the tumor again increased rapidly in size. There was probable evidence of control of tumor growth and definite benefit to the patient.

Case 59. Female, 41 yr., with a diagnosis of multiple myeloma. Treatment consisted of a course of intravenous OPSPA at a dose of 2.0 mg. kg. Cortisone was given for a period of 8 days, but was discontinued because of edema. After an interval of 6 weeks the patient was given OPSPA, 10 mg. wk., intramuscularly. Before treatment the patient's activities had become increasingly restricted because of bone pain, and X rays showed widespread and extensive bony destruction. For a period of approximately 4 months after therapy X rays showed no progression of the disease, and the patient did well and was active for 2 additional months, when progression of the disease resumed. There was probable evidence of tumor effect and definite evidence of patient benefit.

Case 67. Female, 44 yr., with a diagnosis of postresection carcinoma of the ascending colon with ascites. Treatment consisted of a single course of OPSPA with a dose of 2.2 mg. kg. intraperitoneally. Thirty-five days prior to this course of OPSPA the patient had been given 180 mc. of Au^{198} intraperitoneally. Fluid had formed gradually since that date, but it seemed to be forming at a rapidly increasing rate, and it was concluded that further delay was contraindicated. Previous cases receiving radioactive gold showed a decrease in the rate of fluid formation by the thirty-fifth day. On the first day of OPSPA therapy 9600 cc. of ascitic fluid was removed. Since that time the patient's girth has decreased, she has returned to full activity, and has had no further paracenteses for 36 weeks. There is definite evidence of effect on the rate of ascitic fluid formation and of patient benefit. There is some doubt as to the role of preceding therapy in this favorable course.

Discussion

Selection of cases for therapy was a constant problem in this study, as it may be in any chemotherapy program. Many relatively early cases of recurrent or metastatic disease do not present readily available lesions for objective evaluation. Late cases may very likely be unaffected by any form of therapy. There is no certain method for predicting the specific neoplasms that should be tested against any given agent. The number of cases required and the period of follow-up necessary to determine the results of prophylactic therapy make that method impractical in the study of a previously unproven drug. The logic of prophylactic therapy with a drug of known effectiveness, however, seems unassailable if that drug is not associated with significant risk of morbidity or mortality.

Methods of administration. These are arbitrary. There is no reason to believe that the intravenous route is preferable to the intramuscular. It was originally thought that OPSPA might be metabolized rapidly in tissues and that the intravenous or intra-arterial administration might be more effective. Studies with OPSPA-C^{14} in patients[4] suggest that the metabolic fate of OPSPA may be very similar whether injected by the intravascular or the interstitial route. It does appear that intratumoral injection is more effective in man even though this did not seem to be true for animals.[1] In the second phase of our work intratumoral injection was abandoned because it is our feeling that information gained by this method is of little value to the over-all aims of chemotherapy. Intracavitary injection is, of course, a form of intratumoral injection and subject to the same limitations in value. In other words, this method is clinically useful but, once demonstrated, is of little scientific interest.

Our reasons for resorting to the plan of repeated 5-day courses of therapy were based on two facts: (1) supervision of patients, many of whom came to the University Hospitals from long distances, was much more satisfactory; (2) it seems logical that high doses of a chemotherapeutic agent over a limited period of time should offer fewer opportunities for the occurrence of drug resistance such as may result from the development of alternate metabolic pathways or from the primary existence of relatively resistant cells.

The *toxic effects* of OPSPA were, for all practical purposes, limited entirely to the hematopoietic system. It is not evident from the cases presented in Group II of this work that ACTH or cortisone provided any measure of protection. It is our feeling that, although the number of cases is small, the evidence is adequate to determine the absence of protection under the conditions of this study. The delay in appearance of hematopoietic depression presents a significant problem in the administration of OPSPA in persistent therapy. In giving this drug to patients who come to the hospital from long distances, it is difficult to obtain the constant follow-up during 4 or 5 weeks that would be desirable. If hematopoietic depression is prolonged, recovery may be delayed and incomplete Following a single course of therapy, recovery seems to follow the period of maximum depression within a matter of 1 or 2 weeks.

There is no doubt that many patients could tolerate higher dosages of OPSPA than the dosage of 0.5 mg. kg. day × 5 that was arrived at as a safe level in the Group II cases. The problem, of course, is the fact that there is a wide range of sensitivity to the drug, not only in different patients but, apparently, under different circumstances in the same patient.

The *results of therapy* with OPSPA, except for two cases of leukemia and one case of reticulum cell sarcoma, show little significant evidence of effectiveness. This study was primarily concerned with solid tumors, excluding the lymphomas, and the results in this group of patients were uniformly disappointing. The effect upon effusion into serous cavities and upon directly injected lesions should not be compared with effectiveness upon distant sites. If a really valuable chemotherapeutic agent is to be found it must be capable of affecting distant and unapproachable lesions.

The evaluation of cases in which progression of disease is arrested but significant regression cannot be measured is subject to errors in conclusion. Such results should not be ignored, and yet the vagaries of neoplastic disease are such that what is recorded as tumor effect of this sort must be accepted with reservation. Even such measurable lesions as pulmonary metastases, as demonstrated by X-ray films, and the amount and rate of fluid formation may be subject to, and vary with, influences other than the chemotherapeutic agent employed. Finally, not even the diagnosis of neoplastic disease is entirely free of possible error. The case of lymphocytic leukemia, J. L., described in Group I, has experienced such an unexpected course that doubt is naturally cast upon the original diagnosis. In any event, review of the case and re-examination of marrow specimens leaves little reasonable doubt as to the fitness of the diagnosis of leukemia.

What, then, is the present status of OPSPA? It is an interesting compound, historically, because it was synthesized specifically as an antitumoral agent on theoretical grounds based upon previous experience with triethylene melamine (TEM)[6] and triethylene thiophosphoramide (Thio-TEPA).[7] It is an alkylating agent that shows marked value against transplanted tumors in rats and mice and that is tolerated in man at levels capable of influencing neoplastic growth. Under the conditions of this study, OPSPA has not proved to be a valuable drug in the treatment of solid tumors. The effectiveness of OPSPA in treatment of leukemias and lymphomas has been strongly suggested, but the relative value of this drug has not been ascertained in these conditions. There is no evidence to suggest that the effectiveness of OPSPA is influenced by the addition of ACTH or cortisone to the regimen, nor is there any suggestion of protection of the hematopoietic system by these hormones. It is impossible to differentiate, in the cases presented, between the effect of OPSPA and the possible effect of ACTH or cortisone. Several of the results obtained might well have resulted from the hormones alone.

Summary

(1) A clinical study of the alkylating agent OPSPA in 102 patients in Group I and 68 patients in Group II is presented.

(2) Evidence of significant benefit has been shown in 1 case of granulocytic leukemia, 1 case of lymphocytic leukemia, and 1 case of reticulum cell sarcoma.

(3) Evidence of tumor effect in 9 other cases is presented, but the significance of these cases is less clear.

(4) Under the conditions of this study, OPSPA shows markedly limited value in the therapy of solid tumors but, in the opinion of these examiners, is comparable to other alkylating agents reported in the literature.

(5) The relative usefulness of OPSPA in the management of leukemias and lymphomas may warrant further study.

(6) No enhancement of therapeutic value and no protection of the hematopoietic system has been provided by the addition of ACTH or cortisone to the regimen for OPSPA.

References

1. Heidelberger, C. & M. E. Bauman. 1957. Studies on OPSPA. I. The effect of several phosphoramides on transplanted tumors. Cancer Research. **17**: 277–283.
2. Maller, R. K. & C. Heidelberger. 1957. Studies on OPSPA. II. Distribution and excretion of radioactivity following administration of OPSPA-C^{14} and OPSPA P^{32} to the rat. Cancer Research. **17**: 284–290.

3. Maller, R. K. & C. Heidelberger. 1957. Studies on OPSPA. IV. Metabolism of OPSPA in the rat and human. Cancer Research. **17**: 296, 301.
4. Maller, R. K., F. A. McIver & C. Heidelberger. 1957. Studies on OPSPA. III. Distribution and excretion of radioactivity following administration of OPSPA-C^{14} and OPSPA-P^{32} to humans. Cancer Research. **17**: 291–295.
5. McCarthy, W. D. 1955. The palliation and remission of cancer with combined corticosteroid and nitrogen mustard therapy. New Engl. J. Med. **252**: 467–476.
6. Silverberg, J. H. & W. Dameshek. 1952. Use of triethylene melamine in treatment of leukemia and leukosarcoma. J. Am. Med. Assoc. **148**: 1015–1021.
7. Shay, H., C. Zarafonetis, N. Smith, I. Woldow & D. C. H. Sun. 1953. Treatment of leukemia with triethylene-thiophosphoramide (Thio-TEPA). A.M.A. Arch. Internal Med. **92**: 628–645.

CHEMOTHERAPY OF CANCER BY ETHYLENIMINO QUINONES: ITS FOUNDATIONS AND PROBLEMS

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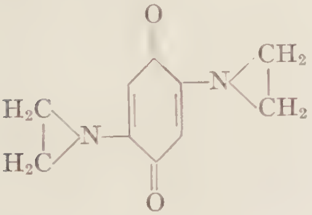
Following the discovery of the curative effect of the sulfonamides in bacterial infections and the recognition that this effect was due to bacteriostasis, it was natural to think that the growth of tumor cells might be inhibited by the administration of systemically well-tolerated chemical substances. L. Heilmeyer called such substances "cytostatic" and distinguished several groups, for example, cell poisons, mitotic poisons, and antimetabolites. Into which group the ethylenimino quinones should be placed is questionable.

In this paper I shall only outline the experimental results obtained with the most effective and best-known substances of this group, namely, 2,5-bis(ethylenimino)quinone and its derivatives, and the results we may reasonably expect from their clinical use.



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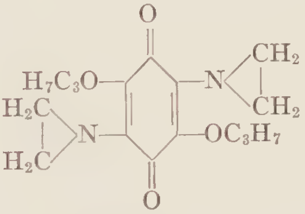

II (Bayer E 39)

In preliminary tests, in which these substances were allowed to act on a tumor-cell suspension refrigerated for 4 hours prior to inoculation, observation of the dilution that eliminates transplantability showed varying results (TABLE 1). With E 39, elimination of transplantability in rat tumors could be demonstrated after only 1 hour with dilutions of 1:10,000 or 1:100,000.

The results of such *in vitro* experiments are, of course, not conclusive with regard to the effects in animals and in man. This we already know from the chemotherapy of bacterial infections in which, on the basis of *in vitro* experiments, one would expect, not the sulfonamides, but the quaternary ammonium bases to be most effective. However, a tentative conclusion may be drawn if the inhibitory effect of the cytostatic substances is considered in relation to the daily dose that can be given to humans. From the inhibition values in TABLE 1, the cytostatic effects of E 39 and TEM appear to be equal. Since it is possible to give humans a daily dose of 40 to 50 mg. of E 39 and only 5 mg. of TEM, a cytostatic concentration is more likely to be achieved with E 39 than with TEM.

In mouse tumors, such as Ehrlich's carcinoma and Crocker's sarcoma, cytostatic values of 1:10,000, or 1:100,000 at best, can be obtained with the technique described above. These preliminary orientating tests show that the various transplantation tumors of animals react quite differently. It is therefore highly improbable that all human tumors behave in the same way. Transplantable carcinomas and sarcomas that respond will do so at various concentrations. For the HeLa strain, a carcinoma of the human cervix, a concentration of 1:100,000 is required in order to achieve an obvious effect within several hours.

Table 1

	Dosage that eliminates transplantability		Maximum daily dose for adults (mg.)
	Yoshida sarcoma	Walker carcinoma	
Nitro-Lost (Mitomen)	1:10,000	1:10,000	20
Triethylene thiophosphoramide (Thio-TEPA)	1:100,000	1:100,000	50
Ethylenimino quinone E 39	1:10 million	1:10 million	50
Triethylene melamine (TEM)	1:10 million	1:10 million	5

Can we hope that it is possible to achieve in the animal body (or even in human tumors) the necessary concentration for a sufficiently long time without killing the animal? This seems to be best effected by injecting into rat tumors of the size of a hazelnut to that of a walnut 0.2 or 0.5 cc. per 100 gm. body weight of a 0.01 per cent solution of Bayer E 39, daily for 10 consecutive days. Under this treatment the tumors regress completely and are no longer demonstrable even by microscopy. In human tumors of equal size similar results may be expected with the same treatment in appropriate dosage. With the rat tumors, when subcutaneous or oral treatment with the above doses is commenced 24 hours after transplantation the tumors may still be prevented from growing. With intravenous, subcutaneous or oral treatment it is even possible in certain cases to cause tumors of the size of a walnut to regress.

Only clinical observations can answer the question of whether, in humans, intravenous administration alone—a therapy successfully introduced by H. J. Wolf and N. Gerlich[1]—or even oral medication, will give the same results.

The rat requires relatively high doses (1 to 10 mg. per kg. body weight) to cause disappearance of tumors that have already developed. However, parenteral or oral treatment achieves complete disappearance within a few days; there remains only the scaffolding of connective tissue, in the meshes of which the tumor cells are completely destroyed. With lower doses another reaction occurs; namely, shrinkage of the nuclei of the tumor cells and the development of histological pictures reminiscent of the degenerating epithelioid nodes seen after treatment with Conteben or isoniazid. In addition, giant cells occur in the tumor and in its marginal zones. These giant cells are obviously of various origins; namely, tumor cells that no longer divide normally and, at the margin, giant absorption cells.

It is not possible at present to decide whether Bayer E 39 should be grouped with the antimitotic substances or with the antimetabolites. At the German Pathology Congress in Hamburg in 1954, I described the effect of E 39 and related substances on mitoses, extending to complete suppression. My co-worker, R. Bierling, has now succeeded in observing an even earlier effect on the cell nuclei, namely on the nucleolus of the tumor cells. In the HeLa strain from human cervical carcinoma, a cloudy loosening and splitting-up of the nucleolus occurs after 5 to 6 hours under the action of Bayer E 39 in a dilution of 1:100,000. Dissolution of the nucleolus and shriveling-up of the cytoplasm follow within 24 hours. This applies to all tumor cells without exception, not only to cells that are in the state of mitosis. It may therefore be possible to separate the described effect on the nucleolus from that on mitosis and to produce compounds that act only on the nucleolus; that is, probably on nucleic acid metabolism.

The morphologic changes described parallel the effect on the special carbohydrate degradation in tumoral metabolism discovered by O. Warburg. In 1954, L. Jühling described in Hamburg the effect of the ethylenimino quinones on glycolysis and respiration. H. Pütter, who supplemented these observations, found that, even when the trials are carefully carried out in tumor ascites fluid, the aerobic glycolysis of the tumor cells is primarily inhibited. H. Holzer also found that it is the oxidative action that the ethylenimino quinones or hydroquinones exert on the SH groups that leads to the inhibition of the triosephosphate dehydrase and, thus, of the aerobic glycolysis. This would confirm the practical importance of Warburg's ingenious work, which has frequently been questioned.

Table 2
Inhibition of Aerobic Glycolysis

	Concentration in moles $\times 10^{-3}$ for 50% inhibition
Nitro-Lost	25
Thio-TEPA	32
Ethylenimino quinone E 39	0.035
TEM	3.0

From Holzer's data (table 2) a remarkable agreement, except in the case of TEM, is seen between the cytostatic effect in rat tumors and the inhibition of aerobic glycolysis via triosephosphate dehydrase. Judged by these values, E 39 would again be by far the most effective with regard to inhibition of triosephosphate dehydrase. Holzer suggests that these cytostatic substances act in the same way as ionizing rays, oxidizing the SH groups of the enzyme, and that thus, by inhibition of triosephosphate dehydrase, the aerobic glycolysis and growth of the tumor are blocked.

Pütter has reported values for inhibition of respiration and aerobic glycolysis in experimental tumors, shown in TABLE 3. It is seen that Thio-TEPA and TEM inhibit chiefly respiration, while E 39 inhibits primarily the aerobic glycolysis. Further thorough studies are required, of course, in order to arrive at a finer differentiation of the mode of action of these and other substances. Reports in the literature thus far have claimed that substances with an ethylenimino group are not effective. A number of such compounds have been described[2] that are distinctly effective in animal tumors and also inhibit the aerobic glycolysis, some of them even to a higher degree than E 39.

TABLE 3

	Process mainly inhibited	Concentration producing 50% inhibition*
Thio-TEPA	respiration	0.0001 M
TEM	respiration	0.001 M
E 39	glycolysis	0.00004 M
E 39 (water-soluble derivative)	glycolysis	0.00003 M

* Read at 120 and 240 min. after addition of the preparation.

We are not sure whether the effect of these compounds on glycolysis is primary or secondary. There is another observation that should be considered: the normal growing tumor cells of the Yoshida sarcoma or the Walker carcinoma contain no glutamine because it is utilized immediately in protein synthesis but, after the initiation of treatment with E 39, glutamine appears, as shown by L. Jühling and K. Eben, indicating inhibition of protein synthesis. Since, if we stop protein synthesis we likewise stop glycolysis, the inhibition of glycolysis by E 39 may be a secondary effect. On the other hand, when there is high glycolytic activity, there is a high production of ATP, which is necessary for protein synthesis; it is therefore possible that glycolysis stops first and then protein synthesis. We are carrying out further investigations in this direction.

For the time being little can be said about the final value of the cytostatic substances available for clinical use. Thus far these substances have proved useful mainly in chronic leukemias. Bayer E 39 has been found by H. Schmidt to be effective in myeloid leukemia and also in chronic lymphatic leukemias and Hodgkin's disease. Chemotherapeutic treatment of Hodgkin's disease is otherwise best carried out with Sanamycin, the first antibiotic with a cytostatic action (C. Hackmann). In chronic leukemia it is often sufficient to give 5 mg. E 39 orally, 3 to 4 times daily or, in obstinate cases, an intravenous dose of 20 mg. According to F. Hesse, these oral doses can be given up to a total of 1000 or 2000 mg. in the course of one year without adverse effects on the blood picture. In one case 6000 mg. was given in the course of 18 months without proved harmful effects. With local application of Bayer E 39, in some cases in combination with intravenous treatment, as introduced by H. J. Wolf and N. Gerlich,[1] the following observations were made: H. Loebell, in Münster, and A. Herrmann, in Munich, saw an effect on sarcomas; and H. Prinz, in Hamburg, observed a favorable influence on some inoperable mammary carcinomas (reported to the Hamburg Association of Physicians). In carcinomatous ascites, intraperitoneal injection of E 39 has also proved beneficial (E. Schöler, in Paderborn). Doses of 50 mg. per day in the prescribed NaCl dilution (that is, 10 mg. in 20 cc. physiological saline) were tolerated by the peritoneum without irritation. Intraperitoneal administration appears to be tolerated better than intravenous injection, and Schöler recommends trials with this mode of administration for the treatment of pulmonary carcinoma with high daily doses. Whether in this case, as with intratumor injections, the undesirable side effects on the white blood cell picture of even high doses are less than with intravenous administration is a question that can be answered only by lengthy experience.

I should like to quote some unpublished case histories of patients treated with E 39 by H. J. Wolf at the Städtisches Krankenhaus, Bielefeld.

Case No. 1. E. K., a 58-year-old man. Early in 1955 a hypernephroma of the left kidney was removed operatively, followed by irradiation. By the end of 1956 there were extensive lung metastases and pleural carcinomatosis, necessitating puncture every second day for removal of the increasing volume of exudate. Treatment involved administration of a total of 450 mg. E 39 (40 mg. daily) and pleural punctures with introduction of 20 to 40 mg. E 39 into the pleural cavity. After only the second puncture the exudate solidified on short standing. After the fourth puncture there was no more exudate. At the conclusion of the treatment the lung metastases had regressed approximately 50 per cent, and the patient felt well and remained under control. Eventually the treatment was repeated. For 4 weeks the patient received simultaneously 55 cc. Sarvinal I. V. daily. His white blood count fell to 800, but was completely restored.

Case No. 2. H. M., a 48-year-old woman. Two years previously she had had a right mastectomy for histologically demonstrated mammary carcinoma. This was followed by intensive X-ray therapy. At the beginning of January 1957 she was admitted in good clinical condition, but her lungs were full of metastases; there were extensive skin metastases of the size of a pea on the skin of the breast. After 480 mg. E 39 intravenously (40 mg. daily) the skin metastases completely disappeared, and the lung metastases regressed as much as a third. The patient left the hospital feeling well, but remained under supervision. Her leukocyte count fell to 1200. There were no complications. At present her blood picture is normal.

Case No. 3. K. L., a 48-year-old man. One year before admission a right hypernephroma had been removed by surgery. He was admitted in December 1956 for carcinoma of the right lung; tumor cells were detected. He was given a total dosage of 360 mg. E 39 I.V. At first, he received injections of 20 mg. A 139 five times a day at intervals of 3 days; there was no solidification of the exudate. Subsequently therapy was changed to E 39, 20 mg. intrapleurally. At the second puncture the exudate solidified; after three more injections of 20 mg. E 39 intrapleurally no more exudate was detectable.

The leukocyte count (1200/cu. mm.) was quickly restored to normal. The patient is under supervision.

Case No. 4. H. B., a 46-year-old man. The patient had an inoperable tumor of the left lung, with small-celled bronchial carcinoma. A total dosage of 400 mg. E 39 (40 mg. daily) was administered intravenously. There was a sharp regression of the tumor, as shown by X-ray films. The patient experienced disappearance of hoarseness and a feeling of well-being. The leukopenia produced (1000/cu. mm.) was quickly restored. Eight weeks after discharge he suffered a relapse. Again, 400 mg. of E 39 was given intravenously, and there was renewed regression of the tumor. Leukopenia (800/cu. mm.) was quickly restored. The patient remains under supervision.

In contrast to these results, Wolf has had no success in the treatment of squamous epithelial carcinoma with ethylenimino quinones.

It is possible that a certain reduction of the leukocytes is even desirable and necessary as an indication of the cytostatic effect, and that one should not be too concerned about a slight reduction of the leukocytes. Perhaps we should even maintain the leukocytes at a low level that is not dangerous to the patient as long as we prevent the development of a dangerous phase. For injections into easily accessible tumors it seems advisable to give 10 mg. of E 39 daily on 5 to 6 consecutive days and then to pause for 1 to 2 weeks in order to watch for the occurrence of changes in the white blood cell picture. Then 20 mg. of E 39 should be given for 5 days and, after another interval, 30 mg. or even 40 mg. In order to avoid injection of too much liquid into the tumor, a clear solution of Bayer E 39 is not necessary, but it is sufficient first to dissolve the pure substance in a few drops of alcohol and to dissolve it further with 1 to 2 cc. saline, instead of the 10 cc. used for intravenous injection. For intratumor injection, a depot effect is even desirable. In pertinent experiments, the primary tumor and also the metastases in the corresponding lymph nodes are destroyed.

In a particularly advanced pavement cell epithelioma that had been unsuccessfully treated with X rays (20,000 r), H. Loebell reported the following results of local treatment. The first effect of E 39 treatment was a complete change of mood; a few days after commencement of the injections of Bayer E 39 or its water-soluble derivative, A 139, or E 39 "soluble," in daily doses of 10 or 20 mg., the apathetic patient, who had lost all interest in life, resumed such activities as eating and reading. A very large carcinoma of the pavement epithelium, the size of an apple, protruded in front of the larynx and impaired the respiration (18 years ago the patient had had a thyreotomy for cancer). After the injection of 130 mg. it declined to skin level and sloughed off in pieces. Unfortunately the patient later died from circulatory weakness during an operation on the larynx for respiratory obstruction.

In this case, biopsy specimens were taken at various intervals. Soon after the commencement of treatment they showed the beginning of regressive changes, with decomposition of the nuclei, as seen also in experimental tumors. The pavement epithelium also showed vacuolation (FIGURE 1); this is best seen at one focus that has reached into the skin epithelium. These distinct vacuolar changes are also present in the metastases, which are surrounded by unaffected normal pavement epithelium. At a very early stage, Feulgen's reaction in the tumor cells had disappeared; this would indicate that under the action of the cytostatic agent the nucleic acids required for the construction of the cell nuclei are no longer formed, as has already been described in experimental tumors.

K. Boshamer recommended as life-prolonging in gastric carcinoma that is either inoperable or unsuitable for radical operation the following simple treatment. One hundred mg. E 39 (pure substance) is dissolved in 40 cc. pure absolute alcohol, this alcoholic solution is poured into 60 cc. of a mixture of raspberry syrup and water in equal parts, and the whole well mixed. Of this preparation, 10 cc. is taken twice daily mornings and evenings between meals. In order to ensure fully effective fresh solutions it is also possible to dissolve 10 mg. of E 39 as usual with 1 cc. alcohol in the ampoule and to give this solution in a glass of brandy or liqueur, as preferred by the patient.

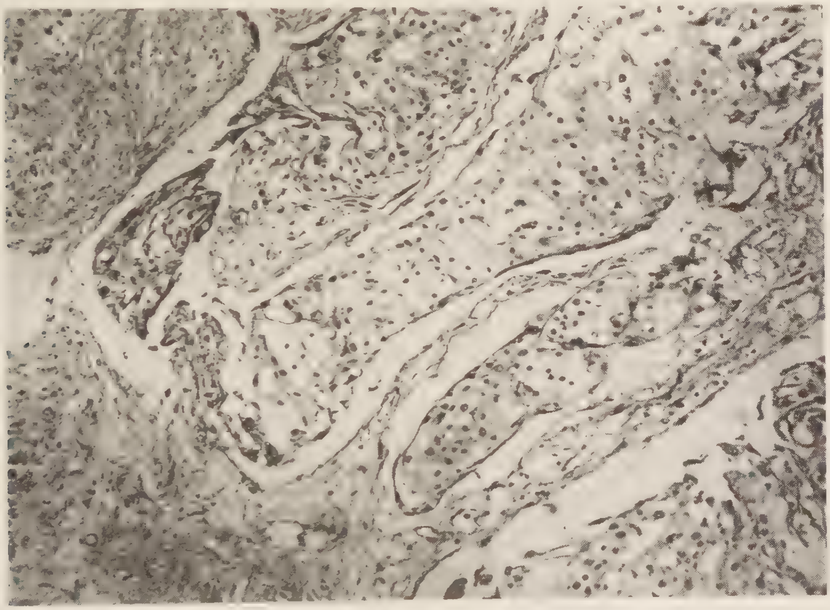

FIGURE 1. E 3535 d. Patient K. Pavement epithelium carcinoma of the larynx. Treated with intratumor injections of E 39 and A 139. Biopsy specimen. Vacuolation in the epithelial cones. Hematoxylin-eosin stain. × 100.

If it were possible to achieve with the intravenous treatment introduced by Wolf and Gerlich or with transfusions a concentration of 1:100,000 in the tumor for at least 24 to 48 hours, we might expect that in human tumors all the cells, without exception, would be destroyed within a few days, as seen in the HeLa cells. Whether in practice we can reach this goal without simultaneously damaging normal tissue cannot yet be determined. In many cases it may be sufficient to convert the rapidly dividing tumor cells to a latent state as in the rare cases of spontaneous cure of malignant tumors and for limited periods after operations. The morphologic indication for such a result effected by Bayer E 39 may be seen in the honeycomb vacuolation of the tumor tissue, the reduction of the cell nuclei, and a hardening of the tumor through increase of connective tissue, instead of complete disappearance of the tumor cells.

Reference

1. Wolf, H. J. & N. Gerlich. 1956. Die klinische Anwendung von Äthylenimochinonen bei Tumorkranken. Deut. med. Wochschr. **81**: 801–811.
2. Gauss, W. & S. Petersen. 1957. Weitere Untersuchungen über Äthylenimochinone und verwandte Verbindungen. Angew. Chemie. **69**: 252–257.

CLINICAL TRIALS OF NONANE IN MALIGNANT DISEASE

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The methanesulfonyloxy alkanes were first studied by Haddow and Timmis in 1953.[1] Subsequent investigations with this group of compounds indicated that Myleran, a C_4 analogue, produced remissions in patients with chronic myelogenous leukemia.[2-12]

Nonane* (1,9-dimethanesulfonyloxynonane) the C_9 analogue of Myleran, was found by Sugiura[13] to have broad antitumor effects in a variety of transplantable animal tumors. Sykes *et al.*[14] have reported clinical benefit in two patients with chronic myelogenous leukemia. For this reason, Nonane was subjected to preliminary clinical trials.

Various dosage schedules were explored to determine the oral dosage comparable in marrow depressant activity to that dosage employed with the usual course of intravenous nitrogen mustard (HN2). The drug was administered orally, once daily, 1 hour before breakfast, in dosages ranging from 3 mg./kg./day to 32 mg./kg./day over periods of from 3 to 20 days (lower dosage level). The dosage schedule arrived at, equivalent to 0.4 mg./kg. of HN2 and which produced a moderate and transient bone marrow depression, was 20 mg./kg./day for 4 days.

Sixteen patients with various malignant neoplasms received a complete course of Nonane therapy (TABLE 1). These diseases consisted of bronchogenic carcinoma (7 patients), chronic lymphatic leukemia (2 patients), Hodgkin's disease (1 patient), carcinoma of the large bowel (2 patients), carcinoma of the testis (1 patient), melanoma (1 patient), and undifferentiated carcinoma (2 patients).

Four patients showed objective improvement; 12 patients were considered therapeutic failures. The objective evidences of improvement were as follows: 2 patients with chronic lymphatic leukemia (decrease in adenopathy and organomegaly, with improvement in peripheral blood and marrow differentials); 1 patient with Hodgkin's disease (decrease in adenopathy and organomegaly); and 1 patient with bronchogenic carcinoma (decrease in pleural effusion).

The following illustrative cases are presented:

Case 1. Chronic lymphatic leukemia. A 42-year-old white male with chronic lymphatic leukemia, which had its onset in January 1956. In August 1956, he received radiotherapy to the spleen with benefit. He entered the hospital in January 1957, with fever, weakness, dysphagia, weight loss, generalized adenopathy (FIGURE 1a), massive hepatosplenomegaly and 4+ bilateral pedal edema. The patient received a total dosage of 80 mg./kg. of Nonane over a period of 6 days. There was progressive decrease in peripheral nodes (FIGURE 1b), decrease in size of liver and spleen, and dis-

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* Provided by Eastman Kodak Co., Rochester, N. Y.

TABLE 1
SUMMARY OF PATIENTS TREATED WITH NONANE THERAPY

Case	Age	Sex	Type	Duration of disease	Previous therapy	Daily dose		Total dose		Hematological toxicity					Response
						mg.	mg./kg.	mg.	mg./kg.	No. days* 1st ↓ WBC	Lowest WBC	No. days* ret. normal	Platelet† depression	Hb fall (gm.)	
1	42	M	C.L.L.	5 mo.	X ray spleen	1000	13.3	6000	80	8	4100	30	+	4	Regression nodes and organomegaly Improved WBC and differentials
2	61	M	C.L.L.	2 mo.	None	1500	20	4500	60	—	—	—	0	1	Regression nodes and organomegaly
3	36	M	H.D.	21 mo.	X ray HN2	750	14	3000	56	10	3600	24	0	2	Regression nodes and organomegaly
4	45	M	Bronchogenic ca.	9 mo.	Myleran	1500	26	6000	104	6	900	50	++	6	Reduction pleural effusion
5	65	M	Bronchogenic ca.	3 mo.	None	1500	16.6	7500	83	10–14	2800	37	+	3	No response
6	63	M	Bronchogenic ca.	3 mo.	X ray	1000 1000	15 15	4000 5000	60 75	20	4800	25	0	2	No response
7	66	M	Bronchogenic ca.	6 mo.	X ray	1500	27	4500	82	—	—	—	0	None	No response
8	39	M	Bronchogenic ca.	4 mo.	Myleran	400 1000	6 15	7200	110	14	1300	—	++	2	No response Died 25th day
9	57	M	Bronchogenic ca.	9 mo.	X ray	750	18	3000	72	13	3300	23	+	1	No response
10	61	M	Bronchogenic ca.	6 mo.	None	1250	20	5000	80	—	—	—	0	None	No response
11	66	M	Large bowel ca.	12 mo.	None	1500	32	4500	96	11	4700	22	0	1	No response
12	60	M	Large bowel ca.	9 mo.	None	1000	20	4000	80	10	2100	—	+	1	No response Died 17th day
13	60	M	Melanoma	4 mo.	None	1000	12.5	4000	50	23	4100	38	+	3	No response
14	41	M	Testis ca.	18 mo.	X ray	200	3	4000	60	20	2200	—	0	2	No response
15	57	M	Undiff. ca. lungs and nodes	15 mo.	None	1000	19	4000	76	9	200	35	++	7	No response
16	25	M	Undiff. ca. peritoneal cavity	22 mo.	None	1000	19	3000	57	9	2600	23	0	None	No response

* From onset of therapy.

† Platelets
0—150,000+
+—100–150,000
++— 50–100,000
+++— 25– 50,000
++++—below 25,000

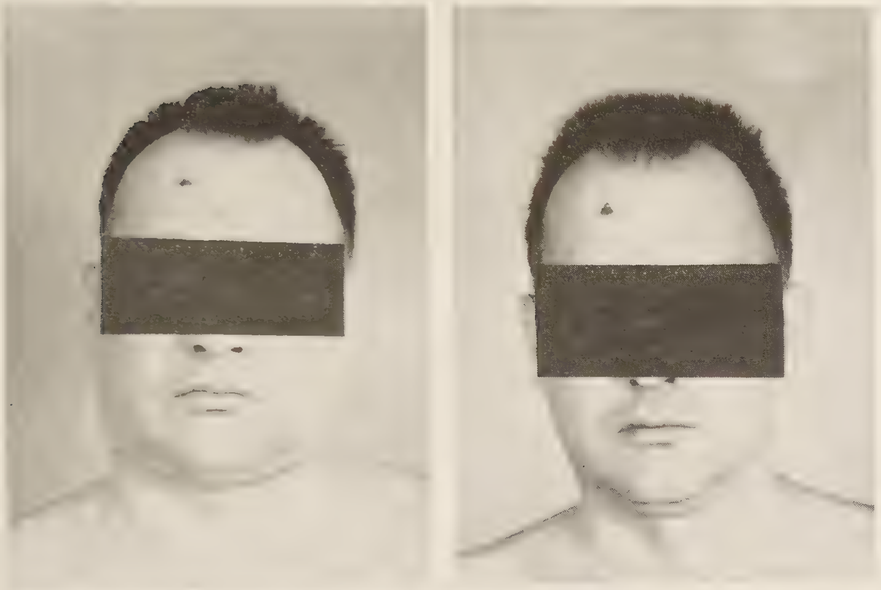

Figure 1. Case 1. Chronic lymphocytic leukemia: (*a*) before Nonane therapy; (*b*) after Nonane therapy.

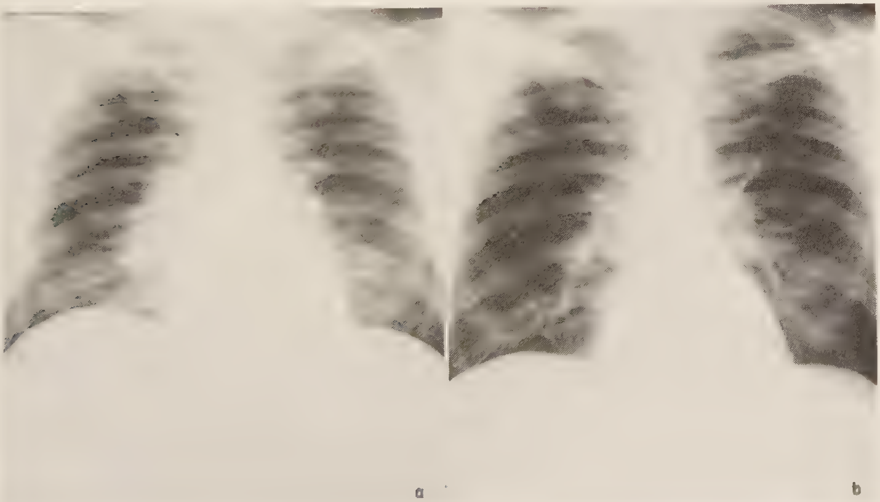

Figure 2. Case 1. Chronic lymphocytic leukemia. Roentgenogram of the chest: (*a*) before Nonane therapy; (*b*) after Nonane therapy.

appearance of pedal edema. Roentgenograms of the chest showed decrease in hilar lesions (FIGURES 2a and 2b). There was improvement in peripheral blood and marrow differentials (FIGURE 3), with a decrease in total lymphocytes and increase in myeloid elements. At present, the patient has returned to work and is in remission.

Case 2. Hodgkin's disease. A 36-year-old white male, whose symptoms began in May 1955. The patient had 3 remissions prior to this admission, having responded to intravenous HN2 and to radiotherapy. He was readmitted in March 1957, with abdominal pain, nausea, anorexia, weight loss, generalized lymphadenopathy, and hepatosplenomegaly. He received Nonane in a total dosage of 56 mg. kg. over a 4-day period. There was disappearance of pain, anorexia, and nausea within a few days, and a marked reduction in adenopathy and hepatosplenomegaly.

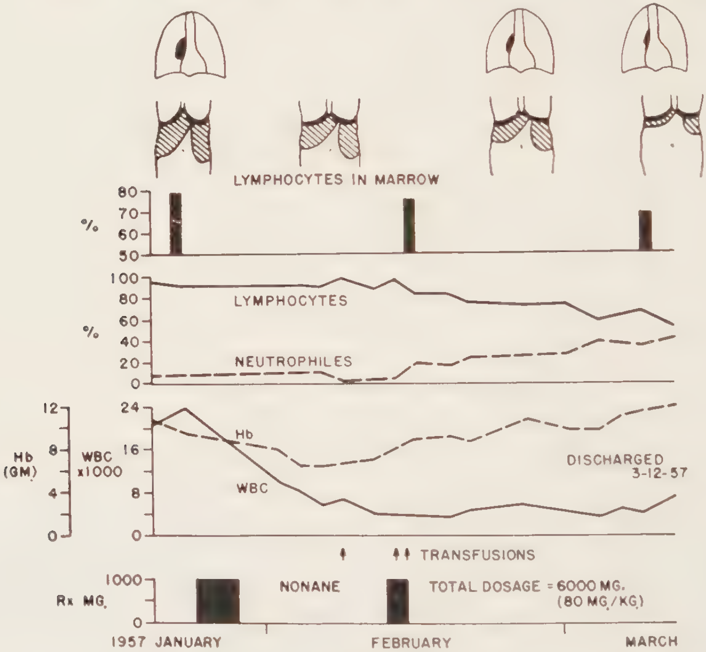

FIGURE 3. Case 1. Chronic lymphocytic leukemia.

Case 3. Bronchogenic carcinoma. A 45-year-old white male who was diagnosed as having bronchogenic carcinoma in April 1956 and who had previously responded to Myleran therapy in June 1956. The response was characterized by decrease in width of the mediastinum. The patient was readmitted in December 1956, with increasing pulmonary disease. He received Nonane in January, 1957, in a total dosage of 105 mg./kg. over a 4-day period. There was decrease in pleural effusion, but residual disease was present. A marked leukopenia, as well as thrombocytopenia, resulted from the rather large dosage of the drug. However, the patient's hemogram reverted to normal by the forty-third day (FIGURE 4). The patient's subsequent clinical course was stationary until 2 months after therapy, when he died suddenly as a result of massive pulmonary hemorrhage.

Twelve patients developed moderate and transient leukopenia, with a slight decrease in hemoglobin and platelet values. Two patients did not develop leukopenia, and showed no change in hemoglobin and only slight thrombocytopenia. Two patients developed marked leukopenia, with considerable decrease in hemoglobin and platelets, accompanied by petechiae and epistaxis. Both of these patients required blood transfusions as adjunctive therapy. In the 14 patients who developed leukopenia (TABLE 1), the onset varied from 6 to 23 days after the start of therapy, with the average at 12 days. The white blood count returned to normal from 22 to 50 days after the start of therapy, with the average at 30 days. One patient developed a transient diffuse alopecia. The compound was well tolerated by all patients and was notable for the absence of untoward gastrointestinal symptoms.

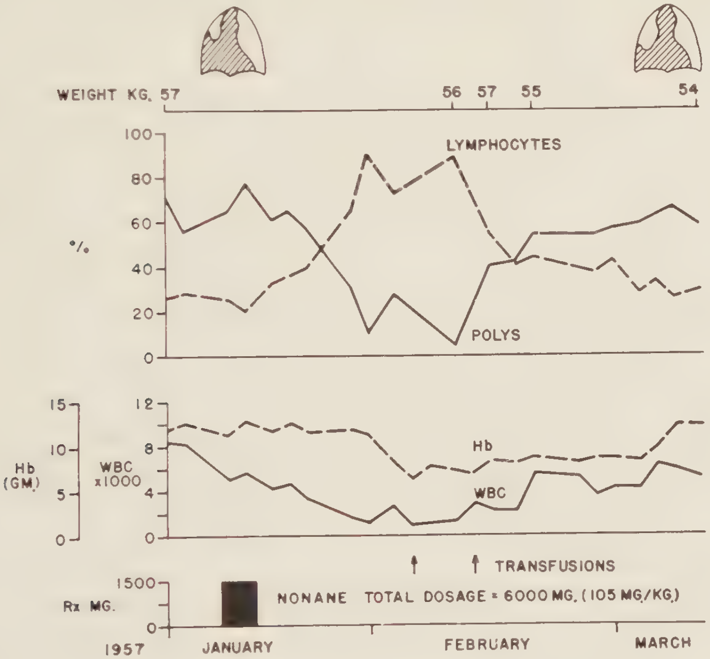

FIGURE 4. Case 3. Bronchogenic carcinoma.

Conclusion

It is concluded that Nonane, in a dosage of 20 mg./kg./day for 4 days, produced bone marrow depression equivalent to the conventional dose of intravenous HN2 (0.4 mg./kg.). Two patients with chronic lymphatic leukemia and one patient with Hodgkin's disease showed objective evidence of tumor regression. One patient with bronchogenic carcinoma showed reduction in pleural effusion. Further clinical studies to compare the therapeutic efficacy of this compound with that of other alkylating agents are in progress.

References

1. Haddow, A. & G. M. Timmis. 1953. Myleran in chronic myeloid leukaemia. Chemical constitution and biological action. Lancet. **264:** 207.
2. Galton, D. A. G. 1953. Myleran in chronic myeloid leukaemia Lancet. **264:** 208.
3. Bollag, W. 1953. Der Einfluss von Myleran auf die Keimdrüsen von Ratten. Experientia. **9:** 268.
4. Bollag, W. 1953. Myleran, a new cytostatic drug in leukemias. Schweiz. med. Wochschr. **83:** 872.
5. Moeschlin, S. 1954. Present status of leukemia treatment. Praxis. **43:** 66.
6. Hansen, P. 1954. Clinical experience with Myleran therapy. especially in myeloid leukaemia. *In* Ciba Foundation Symposium on Leukaemia Research. : 205. Churchill. London, England.
7. Petrakis, N. L., H. R. Bierman, K. H. Kelly, L. P. White & M. B. Shimkin. 1954. The effect of 1,4-dimethanesulfonyloxybutane (GT 41 or Myleran) upon leukemia. Cancer. **7:** 383.
8. Galton, D. A. G. & M. Till. 1955. Myleran in chronic myeloid leukaemia. Lancet. **1:** 425.
9. Videbaek, A. 1955. Myleran (GT 41) in the treatment of leukemia. Acta Med. Scand. **151:** 295.
10. Galton, D. A. G. 1956. The use of Myleran and similar agents in chronic leukemias. Advances in Cancer Research. **4:** 73.
11. Pribilla, W. & G. Stollberg. 1955. The treatment of myelocytic leukemias with Myleran and demecolcin. Deut. med. Wochschr. **80:** 1027.
12. Ramioul, H. 1955. Chemotherapy of myeloid leukemia: 1,4-butanediol dimethylsulfonyl ester (Myleran GT-41). Rev. belge pathol. et méd. exp. **24:** 38.
13. Sugiura, K. 1953. The effect of 6 thiopurine and 1,9-dimethanesulfonoxy nonane on the growth of a variety of mouse and rat tumors. Proc. Am. Assoc. Cancer Research. **1:** 55.
14. Sykes, M. P., F. S. Philips & D. A. Karnofsky. 1956. Comparative therapeutic activity of the nitrogen mustards and allied compounds. Med. Clin. N. Am. **40**(3): 837.

THE INFLUENCE OF 1,4-DIMETHYLSULFONOXY-1,4-DIMETHYLBUTANE (CB 2348, DIMETHYL MYLERAN) IN NEOPLASTIC DISEASE

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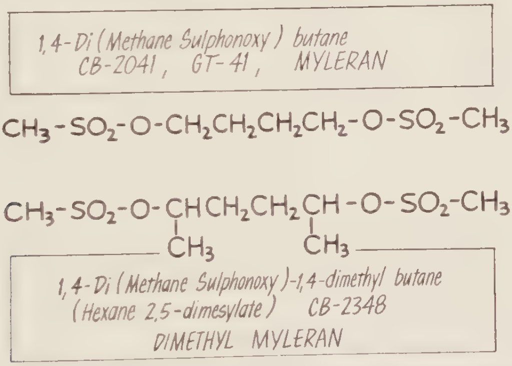
In 1951 Timmis and Haddow[1] introduced the sulfonoxybutanes as effective chemotherapeutic agents in neoplastic diseases. 1,4-Dimethylsulfonoxybutane (Myleran has proved to be a valuable drug in the management of chronic granulocytic leukemia.[2,3] Because of its relative insolubility, Myleran is usually administered orally, and it exhibits its major effect in approximately 2 to 3 weeks. However, in many instances, hematological control with a shorter period becomes imperative. Preliminary studies with an ethanol solution and with an oil suspension of Myleran suggested that parenteral administration might alter its selectivity and activity against various cell types.[4,5] The development of 1,4-dimethylsulfonoxy-1,4-dimethylbutane* (dimethyl Myleran, CB 2348) (FIGURE 1) by Hudson and Timmis[6] permitted the investigation of a soluble sulfonoxybutane in neoplastic disease.


1,4-Di (Methane Sulphonoxy) butane
CB-2041, GT-41, MYLERAN
1,4-Di (Methane Sulphonoxy)-1,4-dimethyl butane
(Hexane 2,5-dimesylate) CB-2348
DIMETHYL MYLERAN

$$CH_3-SO_2-O-CH_2CH_2CH_2CH_2-O-SO_2-CH_3$$

$$CH_3-SO_2-O-\underset{CH_3}{CH}CH_2CH_2\underset{CH_3}{CH}-O-SO_2-CH_3$$

FIGURE 1. Comparison of structural formulas of Myleran and CB 2348.

Patients and Methods

Fourteen patients with various neoplastic diseases received a single infusion of CB 2348 on 19 occasions. Each patient was under careful hospital

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* 1,4-Dimethylsulfonoxy-1,4-dimethylbutane (CB 2348) is a white crystalline powder with a melting point of 94° C. and is slowly soluble in water or alcohol. G. M. Timmis and Alexander Haddow provided the drug in its crystalline form, together with all available data on its characteristics; L. A. Elson reviewed the pharmacological action of the agent in rats; and D. Galton graciously consented to relate his clinical experience in a single patient with chronic granulocytic leukemia treated by the oral route.

TABLE 1

DATA FOLLOWING INTRAVENOUS INJECTION OF CB 2348 IN FIVE PATIENTS WITH CHRONIC GRANULOCYTIC LEUKEMIA

Patient, age, sex	Prior treat	Dur. of disease prior to CB 2348	Dosage		Clinical response	Change in visceral enlargement		Change in blood elements						Remarks
			mg./kg.	mg. total		Spleen	Liver	Packed red cells (PCV)		Platelets × 10³		WBC × 10³		
								From	to	From	to	From	to	
F. A. L. [illegible] F	Myleran	39 mo.	0.4	20	Good	50% decrease	None	26	26	280	190	72	9	No alopecia
B. U. R. 47 M 1st ℞	Myleran	11 mo.	0.5	30	Good	30 cm.* 20 cm.	10 cm. 0	30	36	190	280	70	12	No alopecia Effects 3 wk.
B. U. R. 47 M 2nd ℞	30 mg. CB 2348	13 mo.	0.4	30	Fair	35 cm. 20 cm.	0 0	28	37	160	170	73	6	No alopecia Expired 9 wk. later of bronchopneumonia
R. A. T. 32 M 1st ℞	None	23 mo.	0.5	30	Good	14 cm. 12 cm.	2 cm. 0	28	25	180	200	423	179	Very slight hair loss
R. A. T. 32 M 2nd ℞	30 mg. CB 2348	24 mo.	0.5	30	Good	12 cm. 9 cm.	0 0	35	40	410	220	284	18	Very slight hair loss
L. E. E. 57 F	Surgery for adenoca. ovary 1953. Trans.	7 mo.	0.8	40	Good	5 cm. 2 cm.	2 cm. 0	27	24	150	215	640	4	Total alopecia 3 wk. Returning hair growth in 10 wk.
B. I. C. 74 F	X ray	14 mo.	0.6	40	Good	50% decrease	— —	45	32	N†	420	393	70	No alopecia Expired in 9 wk. Acute myocarditis

All patients were in good condition at infusion of CB 2348. No supplemental treatment was necessary.

* Centimeters below the costal margin.

† Adequate on smear.

observation, and frequent examinations of the blood and bone marrow aspirates were performed by standard techniques.[7] All diagnoses were proved by biopsy or marrow aspiration.

The drug was first dissolved in 2 to 5 cc. of warm 95 per cent alcohol and then diluted to a convenient volume with 0.9 per cent saline solution in a 20 cc. syringe. It was then administered intravenously, slowly so as to avoid venospasm due to the alcohol.

Results

Chronic granulocytic leukemia. Five patients with chronic granulocytic leukemia received a single intravenous infusion of 20 to 40 mg. (0.4 to 0.8 mg./kg. body wt.) of CB 2348 on 7 occasions (TABLE 1).

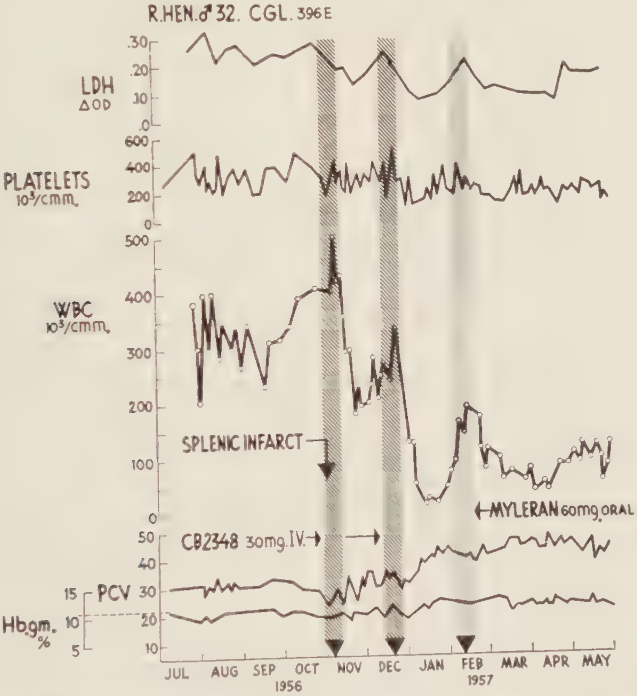

FIGURE 2. Hematological data of a 32-year old man with chronic granulocytic leukemia following two intravenous administrations of 30 mg. of CB 2348 and comparison with one subsequent oral administration of 60 mg. of Myleran. Note the spontaneous rise in platelet number, hemoglobin, and hematocrit. The decreases in circulating leukocytes, primarily at the expense of the immature granulocytes, were rapid and persisted 5 and 8 weeks, respectively, after CB 2348. Following Myleran the response was slower and persisted for 12 weeks.

All 5 patients with chronic granulocytic leukemia were in good condition at the time of treatment and all demonstrated prompt clinical and hematological improvement following a single intravenous injection of CB 2348 (FIGURE 2). There was prompt decrease in splenomegaly, up to 50 per cent, in 4 of the 5 patients. Significant decrease in hepatomegaly occurred in 3 of the 5 patients.

The packed red cell volume and platelet level were maintained or increased in all but one instance without supplementary transfusions. The leukocyte count decreased promptly toward the normal levels at the expense of the immature and mature granulocytes, and the differential leukocyte count gradually shifted toward one predominantly of more mature granulocytes (FIGURE 3). The bone marrow aspirates showed a decrease in cellularity mostly, although not exclusively, in the immature granulocytes within 4 to 7 days.

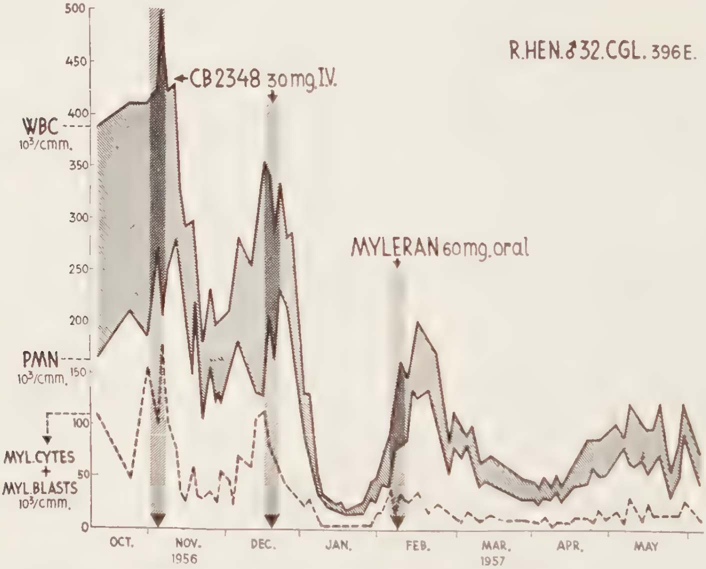

FIGURE 3. Differential leukocyte counts following intravenous administration of CB 2348. The hatched line between the total white blood cell count and PMN represents the immature leukocytes. Note the decrease in immature cells following CB 2348. The dotted line represents the effects upon the myelocytes and myeloblasts. The difference between the myelocyte and myeloblast level and the PMN level represents the metamyelocytes.

The duration of the response varied from 1 to 3 months.

Acute granulocytic leukemia. Two patients had acute granulocytic leukemia and one additional patient had an acute terminal exacerbation of a long-standing chronic granulocytic leukemia when they received a single intravenous infusion of 10 to 30 mg. of CB 2348 (0.4 to 0.7 mg./kg. body wt.) on 4 occasions (TABLE 2).

All of these 3 patients were in poor condition at the time of treatment with CB 2348. Two patients failed to respond clinically in more than a transient fashion, despite the consistent precipitous fall of the peripheral leukocyte level and supportive prednisolone, antibiotics, and blood transfusions.

TABLE 2

DATA FOLLOWING INTRAVENOUS INJECTION OF CB 2348 IN THREE PATIENTS WITH ACUTE GRANULOCYTIC LEUKEMIA

Patient, age, sex	Prior treat.	Dur. of disease prior to CB 2348	Condition at time of treat.	Dosage		Supp. therapy	Clin. resp.	Spleen	Liver	PCV		Platelets $\times 10^3$		WBC $\times 10^3$		Remarks
				mg./kg.	mg. total					From	to	From	to	From	to	
A. U. G. 29 F	Intermittent Myleran Mar. 54–Sept. 56. Colcemide dur. Sept. 56 Transfusions	42 mo.	Terminal	0.4	20	Transfusions	Transient improvement	20–15 cm.*	No change	22	14	180	50	28	2	No alopecia Expired 1 mo. 11/29/56
G. R. O. 67 F	Transfusions	6 mo.	Terminal	0.6	30	Transfusions Prednisolone 15 mg./day	None	No change	No change	19	31	60	30	151	2	Expired 9 days later. 12/29/56
S. M. I. 2 F	Antibiotics Transfusions Methotrexate Meticortin	5 mo.	Moribund Hemiplegia	0.7	10	Transfusions Antibiotics Hydrocortisone 50 mg./day	Hemiplegia cleared 2 wk.	9 cm. 1 cm.	1 cm. 0 cm.	30	30	30	35	27	1	Transient recovery from hemiplegia
S. M. I. 2 F	CB 2348 10 mg.	5 mo.	Moribund	0.7	10	Transfusions Corticosteroids	None	7 cm. 2 cm.	0 cm. 0 cm.	33	26	45	20	5	0.4	Slight alopecia starting on 19th day Expired 1/3/57

* Cm. below costal margin.

The third patient, S. M. I., a 2-year-old girl with acute myeloblastic leukemia, received 10 mg. of CB 2348 8 hours *after* lapsing into a comatose state with a left hemiplegia (FIGURE 4). Within 16 hours the child was awake, alert, and moving all extremities. This dramatic improvement persisted for 2 weeks, during which time the lactic acid dehydrogenase serum level fell to the normal range from an exceedingly high value of over 4.00.[8] Thereafter the condition worsened and, despite a second administration of 10 mg. of CB 2348 and another brief period of improvement, she expired 1 week later. The additional medication, including corticoids, antibiotics, and blood transfusions, was continued from a previous dose schedule that had been unsuccessful.

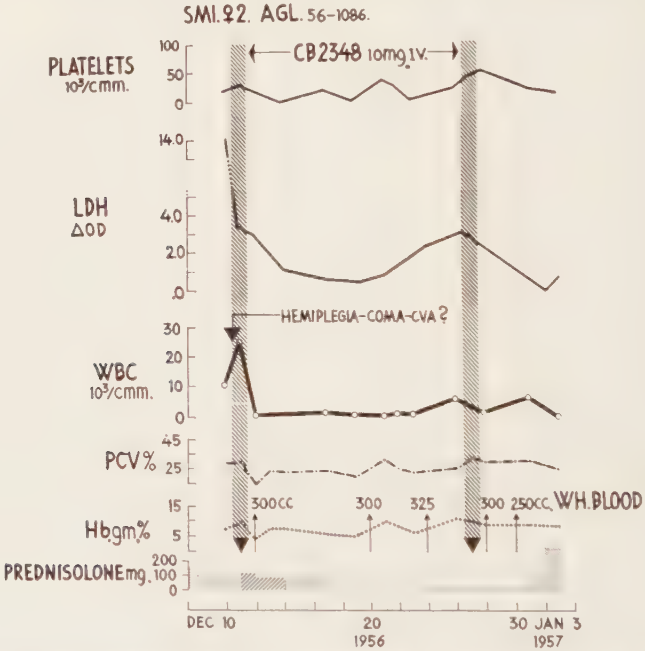

FIGURE 4. Hematological data of a 2 year old girl, S.M.I., with acute granulocytic leukemia following 10 mg. of CB 2348. Prompt hematological and clinical improvement occurred within 16 hours and persisted for 2 weeks. A second 10 mg. infusion was followed by benefit for a week before termination by a cerebral hemorrhage.

Bone marrow aspirates in these 3 patients revealed a profound decrease in total cellularity within 4 days without comparable change in percentage of immature cells. A significant decrease in spleen size was noted in 2 of these 3 patients. The hematocrit continued to decrease in all instances despite repeated blood transfusion. The already thrombocytopenic platelet level fell further following 3 of the 4 infusions of CB 2348. The terminal conditions of each of these 3 patients precludes any adequate evaluation at this time of the effectiveness of CB 2348 in acute granulocytic leukemia.

TABLE 3

DATA FOLLOWING INJECTION OF CB 2348 IN SIX PATIENTS WITH NEOPLASTIC DISEASES

Patient, age, sex	Disease	Prior treat.	Dur. of disease prior to CB 2348	Cond. at time of treat.	Dosage		Clin. Resp.	PCV or Hb		Platelets × 10^3		WBC × 10^3		Toxic effects
					mg./kg.	mg. total		From	to	From	to	From	to	
S. U. N. 61 F	Ca. antrum	Surgery X ray	5 yr.	Terminal	0.2	8 I.V.	None	PCV 30	36*	530	180	4	7	None
B. R. U. 48 F	Ca. of ovary	X ray	10 mo.	Fair	0.6	10 I.P.	Retardation of ascites	Hb 12	10	340	340	7	5	None
B. R. U. 48 F	Ca. of ovary	see above	10 mo.	Fair	1.2	80 I.P.	None	PCV 33	31	385	360	6	3	None
B. R. U. 48 F	Ca. of ovary	see above	12 mo.	Fair	0.5	30 I.V.	None	PCV 34	30	190	230	4	1	None
F. I. N. 54 F	Ca. breast Ca. ovary Duodenal ulcer	Surgery	4 yr.	Fair	0.5	30 I.V.	None	PCV 41	39	460	70	5	1	Alopecia starting on 15th day
T. H. O. 55 M	Chronic lymphocytic leukemia	None	1 yr.	Good	0.6	40 I.V.	None	PCV 35	26	110	70	279	176	Almost complete alopecia in 15 days
G. U. A. 5 F	Ewing's sarcoma	X ray	4 mo.	Poor	0.6	10 I.V.	None	PCV 30	30	350	90	7	2	Alopecia starting on 15th day
B. E. N. 67 F	Ca. cervix	Surgery X ray	20 mo.	Poor	0.7	35 I.V.	None	PCV 21	30*	240	95	22	7	No alopecia in 18 days

I.P. Intraperitoneal route.
I.V. Intravenous route.
* Transfusions.

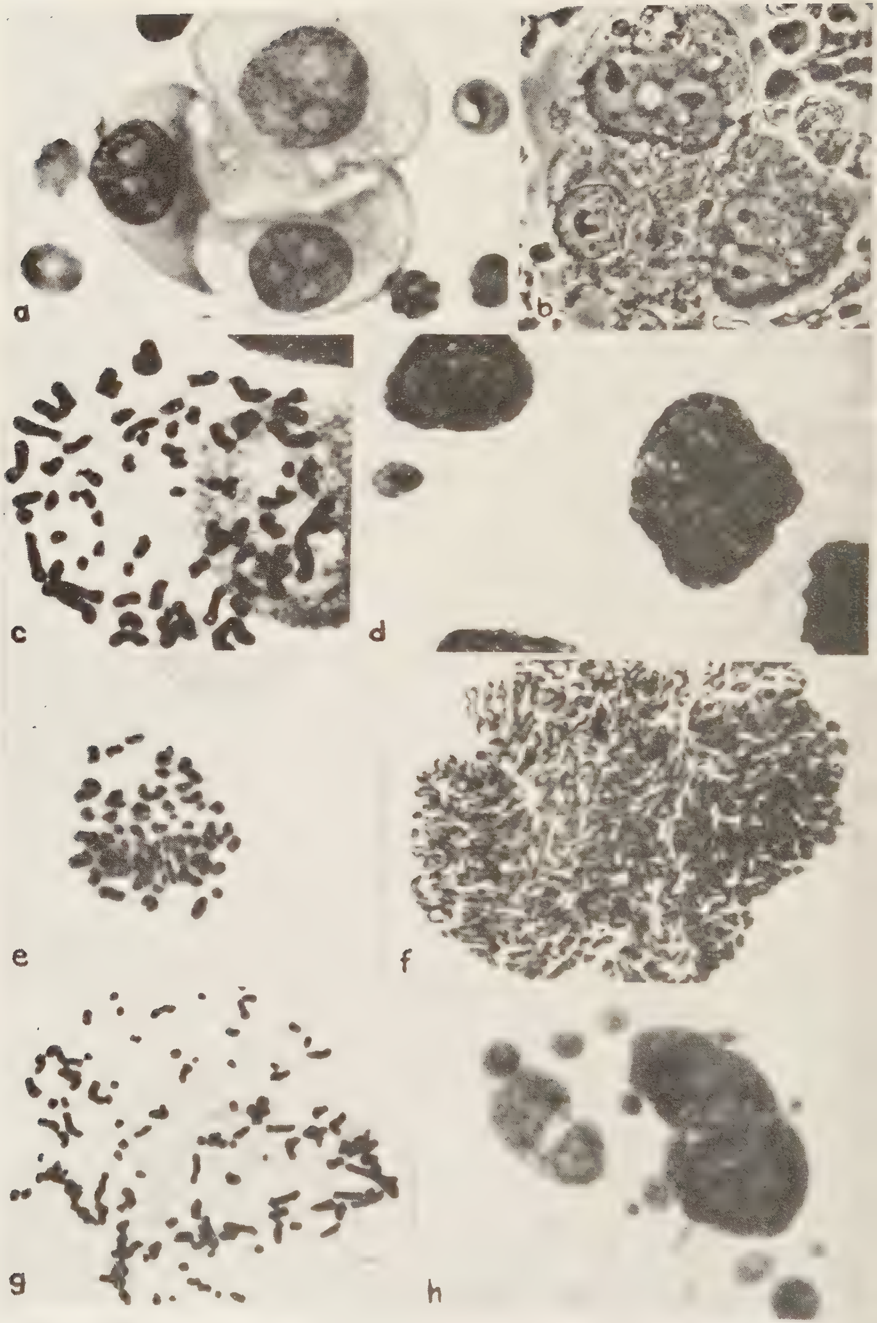

FIGURE 5. Ascites cells from patient B.R.U., suffering from papillary cystadenocarcinoma of the ovary with abdominal metastases.

Before CB 2348: (*a*) three ovarian tumor cells with large nucleoli; (*b*) phase contrast microphotograph of ascites tumor cells; (*c*) normal chromosome complement in ascites tumor cells; (*d*) free tumor cell nuclei in ascitic fluid.

Twenty-four hours after 40 mg. CB 2348 intraperitoneally: (*e*) chromosome clumping; (*f*) abnormal polyploidy; (*g*) chromosome dislocations; (*h*) multiple nuclei of various sizes.

Other neoplastic diseases. The remaining 6 patients had a variety of neoplastic diseases; one of them had chronic lymphocytic leukemia (TABLE 3). Each patient received a single intravenous administration of 8 to 40 mg. of CB 2348 (0.2 to 0.7 mg. kg. body wt.). The peripheral blood leukocyte count decreased in all but 1 of the 6 patients, and the bone marrow showed a predominant granulocytic depressant effect. The lymphocytes were also decreased in patient T. H. O., who had chronic lymphocytic leukemia. The hematocrit was maintained or decreased slightly in each case supported by transfusions (patients S. U. N. and B. E. N.). There was depression of the platelet level in 4 of the 8 instances.

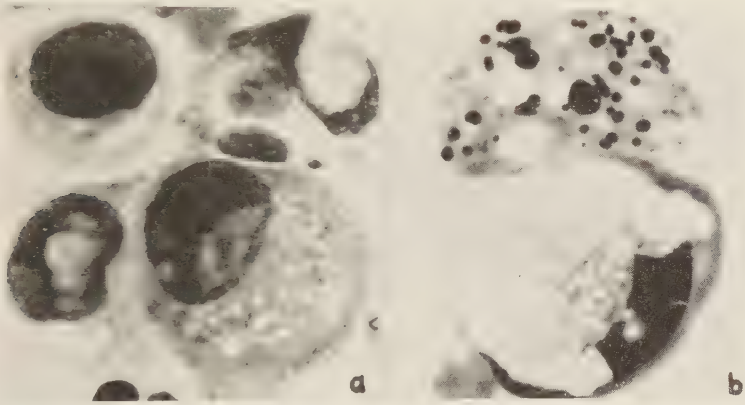

FIGURE 6. Ascites cells from patient S.H.A. with papillary cystadenocarcinoma of the ovary and abdominal metastases: (*a*) prior to treatment; (*b*) 17 hours after intraperitoneal injection of 60 mg. CB 2348—cytoplasmic vacuolation, pyknotic nucleus, nuclear fragmentation, and micronuclei.

One patient, B. R. U., a 48-year-old housewife with adenocarcinoma of the ovary, received two intraperitoneal infusions of CB 2348. Specific nucleotoxic and cytoplasmic effects were observed in the ascites tumor cells (FIGURES 5 and 6). In patient F. I. N. pulmonary hilar enlargement was noted to have decreased significantly following CB 2348, but at this time it is difficult to attribute these changes primarily to CB 2348.

No marked persistent clinical improvement was noted in this group of patients.

Toxicity

Alopecia (FIGURE 7) occurred in 6 of the 14 patients, making its appearance during the third week after intravenous administration of CB 2348 and persisting for an additional 2 to 10 weeks. Thereafter, slow regrowth of the lost hair became evident. Alopecia was encountered only in those patients who received more than 0.5 mg./kg. body weight and was unrelated to the clinical response.

Thrombocytopenia following CB 2348 administration was most severe in the 3 patients with acute granulocytic leukemia. All 3 of these patients

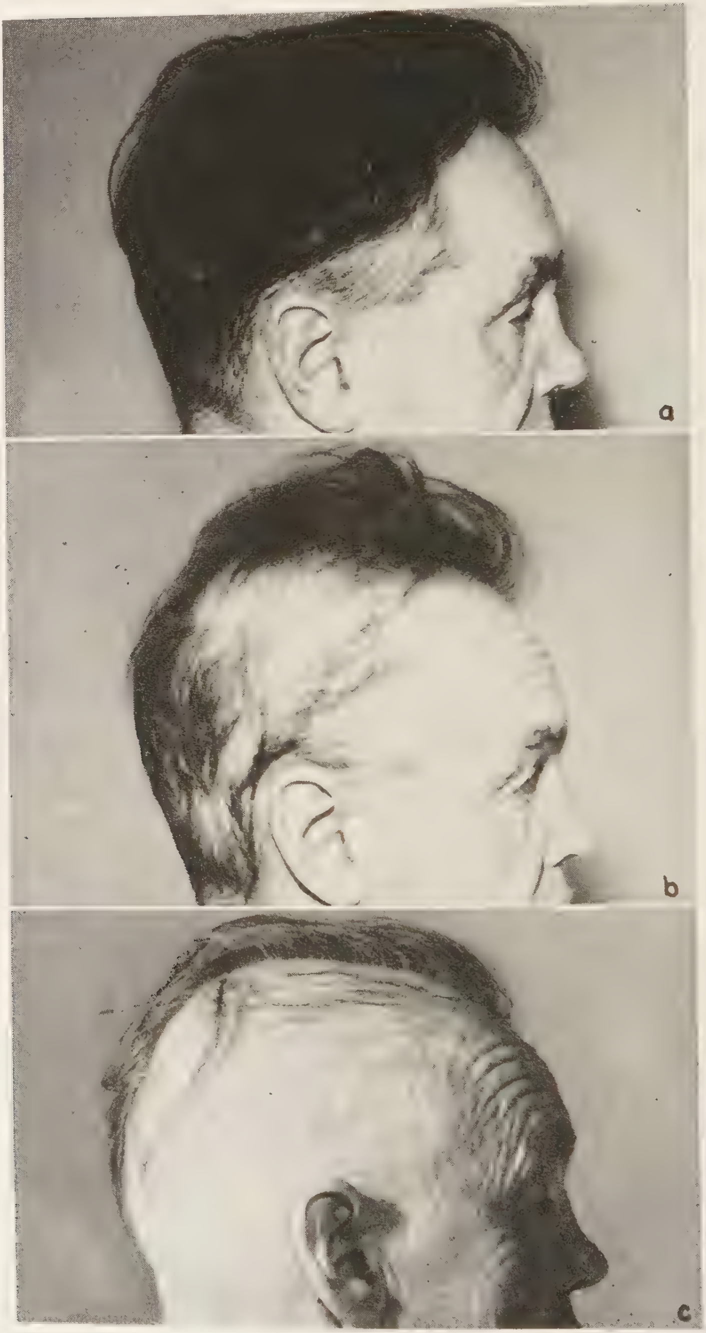

FIGURE 7. Progressive alopecia in T.H.O., a 55-year-old man with chronic lymphocyti leukemia: (*a*) before treatment with CB 2348; (*b*) 21 days after treatment with 40 m CB 2348; (*c*) 24 days after treatment.

had had thrombocytopenia for some time previously and the marrow prior to treatment showed no evidence of megakaryocytosis.

The progressive anemia requiring transfusions in the group of patients with acute granulocytic leukemia and with other neoplastic diseases who received CB 2348 appeared to be the same before and after this medication. The requirement for blood transfusions was unchanged.

Discussion

CB 2348 exhibited a prompt generalized granulocytic depressant effect, more evident in reducing the percentage of metamyelocytes and myelocytes than the polymorphonuclears. In patients with chronic granulocytic leukemia, the decrease in blood and marrow cellularity was observed in 2 to 7 days, as compared with 2 to 4 weeks for Myleran. The beneficial clinical response was supported by the maintenance or rise in the platelet level at the same time as the total leukocyte count, including the number of immature granulocytes in the blood and marrow, was decreasing. The packed red cell volume also increased spontaneously, further emphasizing the significant benefit of the use of this drug in chronic granulocytic leukemia.

The duration of improvement varied from 1 to 3 months following a single intravenous administration of CB 2348. The most satisfactory dose was found to be 0.4 to 0.5 mg. per kg. body weight. Larger doses were followed by temporary alopecia and did not appear to be necessary for attaining a good response in chronic granulocytic leukemia.

In acute granulocytic leukemia, despite the decrease in marrow cellularity in 4 days, the peripheral blood changes were much less profound, and clinical benefit in 3 far-advanced patients was transient. No clinical beneficial effect was observed in 6 patients with other neoplastic diseases.

The production of hematological effects following intravenous administration of CB 2348 appeared to be more predictable than with oral administration of Myleran, perhaps due to the route of administration and the avoidance of gastrointestinal absorptive abnormalities. The intravenous administration of CB 2348 in patients with chronic granulocytic leukemia may be valuable in securing prompt hematological control, within 7 to 10 days, as compared with 3 to 4 weeks for Myleran. In conjunction with oral Myleran, CB 2348 may serve as an effective chemotherapeutic agent in the management of chronic granulocytic leukemia.

Summary

(1) A new cytotoxic agent, CB 2348, has been administered intravenously on 19 occasions to 14 patients with various neoplastic diseases.

(2) All of the 5 patients with chronic granulocytic leukemia showed clinical and hematological improvement for 1 to 3 months following single doses of 0.4 to 0.8 mg./kg.

(3) Two patients with acute granulocytic leukemia and a third in acute exacerbation of a chronic granulocytic leukemia failed to exhibit more than a transient hematological response. In 1 child the improvement lasted for 2 weeks and could not be repeated. The critical stage of illness in these

3 patients precludes any final conclusion as to the ultimate value of CB 2348 in acute granulocytic leukemia.

(4) Six patients with other neoplastic diseases failed to show any significant beneficial responses to a single intravenous infusion.

(5) Temporary alopecia occurred in 6 of the 14 patients within 2 to 3 weeks after the administration of large doses exceeding 0.5 mg. per kg. body wt. Alopecia was not encountered with lesser doses that were also therapeutically effective.

References

1. Haddow, A. & G. M. Timmis. 1953. Myleran in chronic myeloid leukaemia: Chemical constitution and biological action. Lancet. **1:** 207–208.
2. Galton, D. A. G. 1953. Myleran in chronic myeloid leukemia: results of treatment. Lancet. **1:** 208–213.
3. Petrakis, N. S., H. R. Bierman, K. H. Kelly, L. P. White & M. B. Shimkin. 1954. The effect of 1,4 dimethane sulfonoxy butane (GT-41 or Myleran) upon leukemia. Cancer. **7:** 383–390.
4. Bierman, H. R., K. H. Kelly & F. Cordes. 1955. Influence of Myleran upon hematopoietic system in man. Federation Proc. **14:** 319.
5. Tanaka, T., S. Ohno, R. Kinosita & H. R. Bierman. 1955. Effect of Myleran on normal and malignant tissues. Proc. Am. Assoc. Cancer Research. **15:** 51.
6. Timmis, G. M. & R. F. Hudson. 1958. Discussion: Part I. Ann. N. Y. Acad. Sci. 68(3)**:** 727.
7. Bierman, H. R. & K. H. Kelly. 1956. Multiple marrow aspirations in man from the posterior ilium. Blood. **11:** 370–374.
8. Bierman, H. R., B. R. Hill, L. Reinhardt & E. Emory. 1957. Correlation of serum lactic dehydrogenase activity with the clinical status of patients with cancer, lymphomas, and the leukemias. Cancer Research. **17**(7)**:** 660–667.

PRELIMINARY STUDIES WITH SULFUR MUSTARD IN HUMAN NEOPLASTIC DISEASES*

By Louis K. Alpert

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The carcinolytic activity of sulfur mustard, bis(β-chloroethyl)sulfide, has generally been assumed to be identical with that of nitrogen mustard. However, adequate documentary evidence to support this assumption does not appear to be available. Therefore, an investigation of the toxicity and therapeutic effectiveness of sulfur mustard was undertaken to obtain data in this regard.

TABLE 1

Diagnosis	Sulfur mustard Total dose, mg.	Clinical response
Liposarcoma	47.5	None
Ca. rectum	25.0	None
Epidermoid ca., lung	20.5	None
Hodgkin's disease	25.0	Moderate regression for 3 weeks
Chronic lymphatic leukemia	26.0	50% regression in WBC, nodes, spleen, and liver
Reticulum cell sarcoma	10.0	25% regression in nodes, liver, and spleen
Ca. sigmoid	20.0	No objective change Relief of pain
Adenocarcinoma, lung	24.0	No objective change Relief of dyspnea and cough
Ca. stomach	20.0	None
Ca. salivary gland	20.0	50% regression of pulmonary lesions
Melanoma	20.0	None

The sulfur mustard was obtained from the Army Chemical Warfare Service, and prepared by Clarke Davison of the Department of Pharmacology of the George Washington University School of Medicine, in ampoules containing 10 mg. each. Immediately before administration the sulfur mustard was dissolved in 2 ml. of 95 per cent alcohol, and the calculated amount was injected into the tubing of a rapidly flowing intravenous infusion.

During the initial trials, the dosage was started at 1.0 mg. daily and was increased progressively to a maximum of 10 mg. daily. Subsequently, the dosage was approximately 0.1 mg./kg. body weight, usually 5.0 to 6.0 mg. daily, repeated for 4 days.

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* The work on which this paper is based was supported by a grant from the National Cancer Institute, Public Health Service, Bethesda, Md.

The immediate toxic effects were: (1) a sensation of heat or pain in the vein if the solution was injected too rapidly; (2) nausea and vomiting 1 to 3 hours after each injection; and (3) venous thrombosis.

The delayed toxic effects have been related to bone marrow depression, as manifested by a decrease in one or more of the peripheral blood elements approximately 10 days after the beginning of therapy. In the patients who were not terminal and therefore did not die of their malignant disease, recovery of the bone marrow occurred by the end of the third week after treatment was started.

The results are summarized in TABLE 1. Objective regression of lesions was observed in the patients with lymphomas and in one patient with metastatic pulmonary lesions from an adenocarcinoma of a salivary gland.

These preliminary observations have been made in a limited number of patients with far-advanced malignant diseases, and broad conclusions cannot be drawn. However, it would appear that (1) the toxicity of sulfur mustard is somewhat greater than that of nitrogen mustard, and (2) the therapeutic effectiveness of sulfur mustard is generally similar to that which may be expected with nitrogen mustard. Further exploration of this problem is currently in progress.

Part IV. Summary Discussion

COMPARISON OF THE CHANGES PRODUCED BY IONIZING RADIATIONS AND BY THE ALKYLATING AGENTS: EVIDENCE FOR A SIMILAR MECHANISM AT THE MOLECULAR LEVEL

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When it was first discovered that the types of substances discussed in this publication produced, in living cells, end effects that were similar in all respects to those produced by ionizing radiations such as X rays, it was tempting to look for a common primary cause at the cellular level. Perhaps the most striking likeness lay in the genetic changes; for example, a radiation-resistant strain of *Escherichia coli* known as B/r could be obtained by selecting survivors from the wild strain B after exposure to either a dose of X rays or of nitrogen mustard. The B/r mutant, however obtained, showed heightened resistance both to radiation and to the mustard.[1]

The mutagenic action of the nitrogen mustards naturally focused attention on the reaction with deoxyribonucleic acid (DNA). When, in the same year, 1946, Chanutin and Gjessing[2] recorded that the viscosity of dilute solutions of DNA was markedly decreased by treatment with the nitrogen mustard HN2, and Sparrow and Rosenfeld[3] obtained the same effect with X rays, it seemed that ionizing radiations and the alkylating agents acted on the cell by a common chemical mechanism. Since it was known that, in the dilute solutions used for these *in vitro* experiments, the chemical changes produced by the X rays were brought about by the highly reactive free radicals (such as OH˙) formed on irradiating water, the suggestion was made[4, 5] that the mustards also gave rise to free radicals in aqueous solution. However, experiments designed to look for these radicals failed to reveal any evidence for their presence.[6, 7] The need for finding a common chemical mechanism disappeared when Alexander and Fox[8] showed in a model system that, while radiation lowered the viscosity of a polymer by degrading it into smaller molecules, the mustards achieved the same effect by internally cross-linking the molecule so that it occupied a smaller volume in solution. In our other contribution to this monograph[9] further evidence is provided that shows how misleading it can be to deduce an identity of molecular mechanism[10] in reactions that bring about similar changes in viscosity, since these can be the result of entirely different processes.

More detailed studies of the biological changes also revealed differences between the effects produced by radiation and by the chemicals (summarized by Gray[11] and by Bacq and Alexander[12]); these differences suggested that the similarity was confined to the end effects only and that an identity of primary causes cannot be postulated. Our own *in vitro* studies of the reactions of radiations and mustards also led us to this conclusion.[13a, b] However, more recent *in vitro* experiments have caused us to abandon this view, and in this paper we shall summarize evidence suggesting that the biological damage of ionizing radiations and the alkylating agents is initiated at the molecular level by an attack on the same centers, although by different chemical reactions.

Stepwise Development of the Injury

When a cell is irradiated, energy is deposited at random in all its constituents, and subsequent chemical changes will be found at points where they do little harm, as well as at vulnerable points. However, the amount of radiation required to injure mammalian cells is so small that only a few hundred molecules in all will be affected in the nucleus of each cell. The proportion of the low-molecular-weight constituents, such as adenosine triphosphate (ATP) or of the coenzymes, changed will be insignificant when compared with the total amount present. The same consideration probably applies to proteins also; since radiation is nonselective it will attack all the different proteins of the cell indiscriminately and, consequently, the tota out by the observation made by many investigators that if mammalian cell are examined immediately after an irradiation sufficient to kill them some time later, there is either no change or an increase in enzymatic activity.[1 A decrease is observed only if time has been allowed to elapse between irradiation and the measurement of enzyme activity. A reduction in DNA synthesis can be seen as soon as 30 minutes after exposure, while other enzyme systems may not show changes until 48 hours later.

The general pattern by which the radiation lesion develops is clear. Immediately after exposure a chemical change is produced in a very few vita macromolecules (or groups of them). The extent of this initial disturbanc is infinitesimal and will be very difficult to detect analytically. This primary injury is then developed by the cell's own metabolism* to produce biochemical changes such as decreased DNA synthesis in the nucleus or a reductio in oxidative phosphorylation in the mitochondria. These biochemica lesions in turn bring about the anatomical changes that are eventually see by the histologist (FIGURE 1).

Unfortunately, very much less is known of the pathological biochemistr of cells treated with the alkylating agents, and we cannot be certain of th stages that intervene between their administration and the appearance o the damage. Since only very small quantities of the more effective drugs such as TEM (triethylene melamine) or the mustard from L-phenylalanin (CB 3025, sarcolysin), are necessary to damage and kill cells, their actio must follow the alkylation of a few macromolecules that are essential to th cell.† In this initial reaction, not enough of any one type of molecule (c enzyme) will be affected to give rise to biochemical defects directly. Since these alkylating agents are nonselective, they will react to some extent at least with electrophile centers in proteins, nucleic acids, and many other cell constituents. As for radiations, some, if not most, of the reactions that occur are wasted; for example, Roberts and Warwick[14] found that in the rat an appreciable proportion of a compound belonging to the Myleran class combined with cysteine. The loss of cysteine sustained in this way is quite insignificant, and this reaction merely prevents some of the drug from reacting with more important molecules.

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* There is abundant evidence (Bacq and Alexander[12]) that typical radiation injurie are not seen if the organism is maintained after irradiation in a state of reduced metabolis such as hibernation.

† A very crude calculation shows that if we assume that an alkylating agent such a TEM, active at 1 mg./kg., is distributed uniformly throughout the body, then there wi

It is tempting to speculate that the primary reactions of the alkylating agents resemble those of radiation in producing some small defects that do not bring about directly observable changes, either biochemical or anatomical. Enough damage to be detected by ordinary analytical methods is produced only after the cell has tried to function in this faulty condition. Critical experiments to test this hypothesis, similar to those performed with ionizing radiations, could be carried out with alkylating agents such as chlorambucil and dimethyl Myleran which react very quickly (half life at 37° C. is less than 30 minutes). Using these compounds, one could be certain that none of the delayed effects are due to residual amounts of the drugs acting some time after administration.

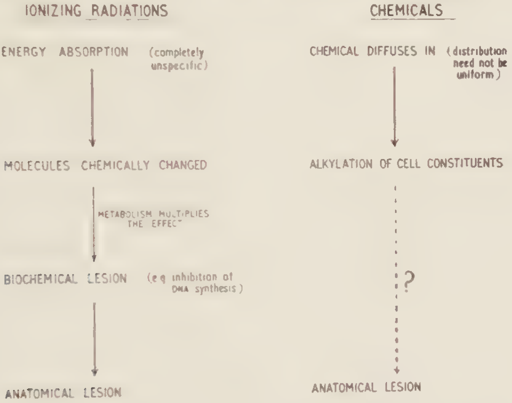
IONIZING RADIATIONS
CHEMICALS
ENERGY ABSORPTION (completely unspecific)
CHEMICAL DIFFUSES IN (distribution need not be uniform)
MOLECULES CHEMICALLY CHANGED
ALKYLATION OF CELL CONSTITUENTS
METABOLISM MULTIPLIES THE EFFECT
BIOCHEMICAL LESION (e.g. inhibition of DNA synthesis)
?
ANATOMICAL LESION
ANATOMICAL LESION

FIGURE 1. Steps in the development of the injury.

The *in vitro* experiments described in this paper indicate that the primary process initiating the cellular damage of both radiation and the alkylating agents is the inactivation of DNA, although the actual chemical reactions responsible for altering the DNA are not the same.

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be 100 molecules in the nucleus (taken to be 1 μ^3 in volume) of each cell. If we further assume the nucleus to be made up of 10 per cent DNA, 10 per cent protein, 10 per cent low-molecular-weight compounds capable of being alkylated, and that all of these consume an equal amount of the alkylating agent, then 30 out of a total of 10^4 DNA molecules will have reacted, while the figures for proteins and other substances alkylated will be of the order of 30 in 10^6 and 30 in 10^8, respectively.

Relative Rates of Reaction of Alkylating Agents with Different Cell Constituents

One of the most remarkable features of the alkylating agents, in particular the nitrogen mustards, is that active compounds are obtained from almost any type of molecule as long as it carries the alkylating centers. Basic, acidic, and neutral compounds, heterocycles, aliphatics, and aromatics all show biological activity. Moreover, compounds that are active at very low doses, as well as relatively inactive compounds, are found in each of the different categories. As an example, we need consider only three of the most active nitrogen mustards: under physiological conditions, chlorambucil is negatively charged, CB 3025 (L-phenylalanine mustard, sarcolysin) is uncharged, while HN2 carries a positive charge. To obtain an indication as to which of the many possible reactions are likely to be biologically important, a number of mustards were reacted with different macromolecules. The relative rates were expressed as competition factors using the formula:

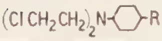
COMPOUND 	BIOLOGICAL ACTIVITY	SERUM ALBUMIN	SERUM GLOBULIN	FIBRINOGEN	DNA	POLY-METHACRYLATE	POLY-ETHYLENE-IMINE
R							
$CH_2 \cdot CH \cdot (NH_2) \cdot COOH$ (PHENYL ALANINE MUSTARD)	+ + +	26	23	—	180	2100	21
$O \cdot (CH_2)_2 \overset{+}{N} \cdot (CH_3)_3 Br^-$	—	40	7	2	460	1400	20
$(CH_2)_2 N\ (CH_3)_2$	+ +	20	—	—	310	4400	—
$(CH_2)_2\ NH_2$	+ +	25	—	—	190	2000	—
$-COOH$	+	31	20	5	0	0	240
$(CH_2)_3\ COOH$ (CHLORAMBUCIL)	+ + +	74	20	20	20	100	170

FIGURE 2. Competition factors of aromatic nitrogen mustards for proteins, DNA, and a positively and negatively charged polyelectrolyte.

$$\text{Competition factor} = \frac{\text{Amount of mustard reacted with macromolecule}}{\text{Amount of mustard reacting with water}} \times \frac{1}{\text{Concentration of macromolecule}}$$

(the concentration was expressed in moles per liter of groups capable of alkylation).

FIGURE 2 shows that there are remarkable differences among the compounds and that there is no correlation between biological activity and the competition for any one macromolecule. While the phenylalanine mustard combines very readily with DNA, the biologically highly active chlorambucil reacts hardly at all with DNA, but combines readily with protein. On the basis of these results one would predict that in the cell, where proteins and nucleic acids are in great excess and compete for a small quantity of mustard, only a minute proportion of chlorambucil would react with DNA, the vast majority being consumed by the proteins. With the phenylalanine mustard the reverse would be the case, and on a weight-for-weight basis DNA would react with more mustard.

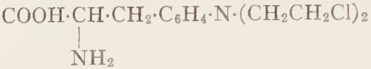
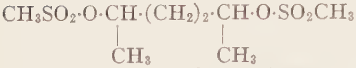
Experiments with model polymers, as well as with chemically modified proteins, showed that all the mustards that carried a positive charge reacted much more readily with negatively charged polymers (such as DNA) than with positively charged polymers. The reverse is the case with negatively charged mustards such as chlorambucil; this compound fails to react with DNA in solution because of the electrostatic repulsion between the phosphate groups in DNA and the ionized carboxyl group in the mustard.

The high biological activity of dimethyl Myleran was also difficult to reconcile with *in vitro* experiments, since this substance did not react at all with proteins or nucleic acids in dilute solutions. Under these conditions dimethyl Myleran was completely hydrolyzed by the water, which was in excess, and its competition factors for all the macromolecules shown in FIGURE 2 are 1 or less. Combination with protein, for example, could be measured only if the protein was present at a concentration of at least 5 per cent.

Clearly, dilute solutions of macromolecules are quite inadequate as models, and the experiments with dimethyl Myleran suggested that systems in which the substrate was present at much higher concentrations should be used. The solid content of subcellular structures such as the nucleus or the mitochondria, where reaction is most likely to occur, is high and, in mammalian tissues, it ranges from 10 to 20 per cent. Under these conditions even substances such as dimethyl Myleran with a low competition factor react extensively with the cell constituents and are not all lost by hydrolysis.

These considerations led us to examine the reaction of the alkylating agents with nucleoproteins. So far most of our data[9] have been obtained with sperm heads from herrings; these are made up entirely of nucleoprotamine (31 per cent DNA and 69 per cent protamine). All the alkylating agents reacted readily with the phosphate groups of the DNA, which appear to be completely accessible to these compounds. In the case of the L-phenylalanine mustard it could be shown again that the reaction was confined entirely to esterification of the phosphate groups. As a result of these reactions the DNA molecules became crosslinked and the DNA was rendered insoluble in 2 M sodium chloride. From TABLE 1, it can be seen that this is a very efficient reaction and that small quantities of mustard can "gel" relatively large amounts of DNA, which will then be useless to the cell. Of greatest significance, however, is the fact that chlorambucil, which does not readily react with DNA in solution, esterifies its phosphate groups in the nucleoprotein. Dimethyl Myleran also reacts with the nucleoprotamine and converts the DNA into an insoluble gel.

TABLE 1
CROSSLINKING OF DNA IN SPERM HEADS

Alkylating agent used	Percentage phosphate groups esterified	Fraction of DNA rendered insoluble in 2 *M* NaCl*
 $\underset{NH_2}{COOH\cdot CH}\cdot CH_2\cdot C_6H_4\cdot N\cdot(CH_2CH_2Cl)_2$	2.6	16
	7.7	83
CB 3025	14.3	91
 $CH_3SO_2\cdot O\cdot \underset{CH_3}{CH}\cdot(CH_2)_2\cdot \underset{CH_3}{CH}\cdot O\cdot SO_2CH_3$	3.0	76
dimethyl Myleran		
$COOH\cdot(CH_2)_3\cdot C_6H_4\cdot N(CH_2CH_2Cl)_2$	3.4	38
chlorambucil	10.5	84

* Insolubility was defined as DNA removed by centrifuging at 20,000 g for 1 hr. If the dispersion of the sperm heads is carried out carefully no DNA from untreated sperm heads is removed under these conditions.

Our *in vitro* experiments led us, therefore, to the view that the biologically important reaction of the alkylating agents occurs with DNA (or possibly RNA[9]) in nucleoproteins and modifies these in such a way that they become useless to the cell. To do this, isolated esterifications are relatively ineffective (Zamenhof *et al.*[15]), and a large number of alkylations by monofunctional agents are necessary. However, the introduction of cross links immediately destroys the biological integrity of the material, and this is why the polyfunctional agents are so effective.

Effect of Radiation on DNA

Since the original discovery of Sparrow and Rosenfeld[5] that DNA is degraded when irradiated with X rays in dilute solution, this reaction has been studied in detail by Taylor, Greenstein, and Hollaender[16] and by others. Under the conditions used, the action of the radiation was entirely indirect; that is, the free radicals formed in irradiated water attacked the dissolved DNA molecules. Direct action requires absorption of the energy by the DNA molecules themselves. In view of the high water content of most cells it seemed reasonable that indirect action should play a major part in promoting cell injuries. This view was reinforced[11, 12] by finding that radiation damage of cells could be influenced by altering external factors such as oxygen tension, by adding protective agents, or by changing temperature. These factors, it was believed, could not alter the effect of direct action since this is a purely physical process. However, more recent experiments[17a, b, c] have proved this deduction false and have shown that direct action is dependent on the environment as much as is indirect action. Since direct action is very much more effective, dose for dose, than indirect action,* the great preponderance of water is more than compensated, especially when it is realized that the local concentration of proteins and other cell constituents in subcellular structures is much higher than the average over the whole cell. In the nucleus, for example, the nucleoprotein gel may have a solid content of 20 to 30 per cent.

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* Lea *et al.*[18] found that tobacco mosaic virus was inactivated one thousand times more efficiently by direct than by indirect action. The ratio is approximately 10:1 for the inactivation of many enzymes other than those containing —SH groups—and for the denaturation of proteins and for breaking the main chain of DNA.[20]

All these considerations lead us, therefore, to the conclusion that the important processes are those produced by direct action.

When we irradiated solid DNA (about 20 per cent moisture content), it appeared to be remarkably radiation-resistant as judged by physicochemical criteria, and it did not seem likely that it would be the center in the cell most vulnerable to radiation. This resistance, we now know, is due to the characteristic structure of the DNA molecule, which Crick and Watson showed to consist of two polynucleotide chains held together by regular hydrogen bonds. A reduction in the molecular weight of this aggregate requires that two breaks be produced very close together in each of the chains (FIGURE 3). As a result, very many of the breaks produced by irradiation with X rays are hidden and can be revealed only after separating the two strands. This can be done by dissolving the DNA after irradiation in a concentrated solution of urea.[19a, b] All the breaks can then be measured, and we found that one of these was produced* for every 20 eV of energy that had been deposited by the irradiation.[20]

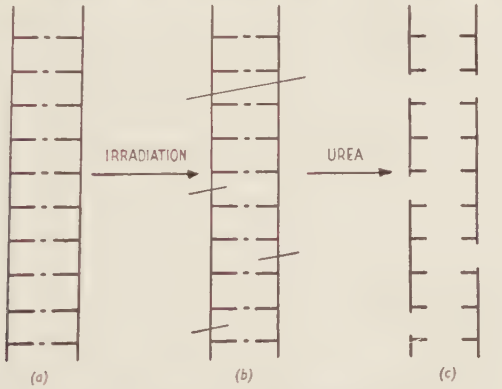

FIGURE 3. Diagrammatic representation of the effect of urea on irradiated DNA, showing how breaks hidden in the dimeric structure become apparent in urea solution when all the hydrogen bonds are broken. (Reproduced from Alexander and Stacey[20] by permission of the authors and the publisher.)

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* One double break (that is, a rupture of the twin molecule) is produced by sparsely ionizing radiations for every 200 eV. With densely ionizing particles such as α rays only double breaks are produced, and these are not hidden breaks. This is to be expected, since along the track of an α particle one ionization is produced every 3 Å., so that on passing through a DNA molecule several ionizations will be produced in each of the twin molecules. Fast electrons, on the other hand, produce ionizations that are hundreds of Å. apart, and they will therefore be randomly distributed between the two strands when DNA is irradiated. The frequency of double breaks with X rays, although low, is still greater than would be expected from chance distribution of ionizations, and they may be produced by the densely ionizing tail end of the electron track.

Using this value, a simple calculation shows that a dose of 1000 r will cause a break in one of the chains of at least 3 per cent of all the DNA molecules present if an average molecular weight of 6×10^6 is assumed. This value is probably too low, since transfer of energy initially absorbed in the protein moiety of the nucleoprotein to the DNA may increase the number of breaks produced. Preliminary experiments indicate that DNA is damaged to a greater extent when irradiated in the herring sperm head than when irradiated by itself. Breaking the main nucleotide chain is likely to prevent a DNA molecule from exercising its normal biological function—such as acting as a template for further DNA synthesis - and it may be useful as a working hypothesis to consider that this reaction is responsible for initiating the processes resulting in the observed radiation lesions in the cell.

Rupture of Polynucleotide Chain by the Alkylating Agents

We have already presented data[9] that confirm the prediction of Brown and Todd[21] that the triester formed on esterification of the primary phosphate groups of DNA is unstable and hydrolyzes slowly under physiological conditions to a diester. Analysis of the hydrolysis products showed that the rate at which the mustard ester hydrolyzes is at least ten times as great as that of one of the nucleic-acid sugar esters. Occasionally, however, the triester opens to produce a break in the main polynucleotide chain. A reaction of this type would be indistinguishable from an interruption of one of the strands by radiation. It would seem possible, therefore, that the alkylating agents and radiation inactivate DNA by the same mechanism. We considered the possibility that the polyfunctional compounds might produce double breaks by reacting with adjacent phosphate groups. In the Crick-Watson model for DNA, neighboring phosphate groups belong to different chains. If both the triesters formed by a bifunctional alkylating agent hydrolyzed at the sugar ester, the twin structure would be broken. The greater effectiveness of the polyfunctional compound could then be ascribed to the more harmful consequences of double as opposed to single breaks.

The data summarized in TABLE 2 do not lend support to the view that the alkylating agents act by producing main-chain breaks. Hidden breaks are produced by treatment with propylene oxide, since the molecular weight measured in urea (which splits the double chain) has fallen, while the molecular weight measured in salt solution has hardly been affected. However, even after standing for 24 hours at 37° C., only one main-chain break has occurred for every 100 groups that had been esterified. Blocking this number of phosphate groups would probably suffice by itself to inactivate a DNA molecule without requiring the production of a break. Moreover, many radiomimetic effects were seen a few hours after treatment, by which time only a very small fraction of the triesters formed would have hydrolyzed, and the probability of a break being produced in the main chain would be low.

The number of "single" breaks produced by treatment with the polyfunctional compounds cannot be determined since, by linking the twin chains together at a number of points, these agents interfere with the dissociation by urea. With the techniques available it is not possible to analyze the

Table 2
Production of "Single" and "Double" Breaks in DNA

Reagent used	Per cent esterification	Molecular weight × 10^6	
		In salt soln.	In urea soln.
None	0	6–6.4	3–3.5
Propylene oxide			
24 hr. at 0° C	49	6.7	~0.2
24 hr. at 37° C		3.3	<0.1
Butadiene bisepoxide			
24 hr. at 0° C	42	7.4	3.2
24 hr. at 37° C		2.15	0.77
L-Phenylalanine mustard			
24 hr. at 0° C.*	3	6.0	2.85
24 hr. at 37° C		6.0	2.25
24 hr. at 0° C	20	5.4	1.8
24 hr. at 37° C		3.05	1.1
24 hr. at 0° C	27	2.7	1.05
60 hr. at 37° C		0.15	—
24 hr. at 0° C	53	0.8	—

* Even this small degree of esterification was sufficient to bring about appreciable internal crosslinking.

changes produced when an internally crosslinked DNA is treated with urea. The fact that the ratio of the molecular weight in the absence and presence of urea remains approximately 2, even after extensive esterification by the bifunctional reagents, does not mean that no "single" breaks have been produced, but that cross links joining the two chains together have compensated for some of the single breaks. Although the two chains in the DNA molecules have been linked by bonds that are not split by urea, some dissociation is to be expected since the formation of single breaks will enable parts of the molecule to separate.

"Double" breaks can be detected with less ambiguity from the change in molecular weight of the double molecule. Table 2 shows that a reduction in molecular weight of the undissociated molecule (that is, the production of "double" breaks) is seen only after very extensive reaction followed by 24 hours for hydrolysis. Smaller amounts of bifunctional esterification that are sufficient to distort the whole shape of the molecule do not reduce the molecular weight. On comparing the efficiencies with which the polyfunctional agents change the shape of the molecule with the production of breaks in the polynucleotide chain, it seems probable that inactivation of the DNA follows from the first reaction rather than from the second. With the monofunctional compounds it is more difficult to distinguish between the possibility that inactivation is the result of blocking a number of phosphate groups or of producing a main-chain break, although we are inclined to favor the former mechanism.

To summarize: our experiments suggest that, while radiation inactivates DNA by producing main-chain breaks, the polyfunctional alkylating agents produce the same effect by producing either intermolecular or intramolecular cross links, depending on conditions.

The Importance of Nucleophilic Alkylation

The DNA "deletion" mechanism explains why only the nucleophilic alkylating agents have radiomimetic properties. Many other types of reagents that can combine with cell constituents under physiological conditions (for example, by alkylation or by acylating NH_2 or SH groups) have been examined and found inactive. All these substances react readily with proteins and profoundly modify them; if radiomimetic action depended on the inactivation of proteins, these reagents should be at least as effective as the nucleophilic alkylating agents. In DNA, however, the only readily accessible group by which the molecule can be attacked is the phosphate group. The amino and imino groups and the ring nitrogens are prevented, either sterically or as a result of hydrogen bonding, from reacting, and none of the acylating agents that readily combine with proteins under very mild conditions [for example, dinitrofluorobenzene or thiol acetate (—CO·S·R—)] can be made to do so with DNA.[22a, b] The reaction of epoxides with amino groups in DNA probably occurs only after the molecule has been severely degraded following esterification. The only way by which preformed DNA can be modified chemically in the cell is by reaction with the phosphate groups, which appear to constitute the Achilles heel of this vital material; and the only reagents that react with anions under physiological conditions are the alkylating centers that have been found to confer biological activity on molecules.

If, indeed, one were to set out to find methods by which to attack DNA, one would inevitably end with radiations and the nucleophilic alkylating agents.

Although the actual reaction of the alkylating agents with DNA is probably quite random and purely destructive in the sense that it renders the molecule useless to the cell, it may prove possible to design alkylating agents that react preferentially with the DNA in one kind of nucleoprotein rather than another. For example, mustards carrying a positive charge will react preferentially with a nucleoprotein that has an over-all negative charge, while the reverse will apply to cationic reagents such as chlorambucil. In this way differences between different compounds may appear even at the cellular level, and the possibility of obtaining greater reaction with tumor than with normal cells is by no means excluded.

Selective action within the cell is, of course, a different problem from the localization of the drug in different organs, on which probably most of the observed selectivity of the various drugs—such as Myleran—depends.

Difficulties in Facts and Interpretations

There must be differences between the effects produced in the whole animal by the chemicals and by ionizing radiations, even if their mechanism at the molecular level is the same, because not all cells will be exposed to the same concentration of drug, while exposure to radiation will be nearly uniform. Qualitative variations in the effects produced within the cell can probably be explained also by differences in distribution. For example, material next to the cell (or nucleus) membrane will have a greater chance of combining with the chemical alkylating agents, which must diffuse into the cell from outside, than will similar substances at the center. No such difference would be found with ionizing radiations. A diffusion effect might be responsible for the concentration of breaks in certain parts of the chromosomes found in some cells (for example, in cells of *Vicia faba*), although by no means in all, after treatment with the alkylating agents, but not after irradiation, which produces essentially random breaks.[23] Differences in the relative frequency of mutations produced by the chemical and by radiation could also be brought about in this way.

Another factor that might influence the end effects is the time taken by the chemicals to react in the cell. In the case of radiation, all the primary chemical reactions will be complete within microseconds after the exposure, while the chemicals may persist and continue to react for hours, if not days, after administration. This delay could be responsible for the absence of an "intensity" effect with the chemicals, to which Revell has drawn attention[23] and for the occurrence of delayed mutagenic changes, which give rise to mosaics.[24]

A more powerful reason for rejecting a common mechanism is the observation originally made by Koller and Casarini[25] and by Revell[23] that no chromosome abnormalities are produced when the cell is treated with the chemicals toward the end of the resting stage, while irradiation is effective at any time during the resting stage. More recent work[26] has shown that the differences in the sensitivity are not as marked as had at one time been believed and that temporary arrest of mitosis complicates the interpretation. It would appear, however, that a distinct difference in timing remains to be explained. It may be that the chromosome material consolidates during the end of the resting stage and that the DNA is rendered inaccessible to the chemicals while still responding to radiation.

Perhaps the most serious difficulty of the DNA "deletion" hypothesis is the production of mutations that cannot arise by loss. None of the mechanisms proposed could bring about a rearrangement in the purine-pyrimidine sequence, which is the most likely way of transmitting the gene code. It is reasonable that crosslinking and breaking of polynucleotide chains should spoil DNA, but neither of these processes would appear to lend itself to producing the subtle changes required for altering the function of a gene.

Finally, it must be emphasized that many of the most characteristic features of radiation damage, such as the oxygen effect, and the variation in effectiveness of different radiations do not follow directly from the damage to DNA. These factors may not influence the primary step and may enter only into the complex interplay of the processes of repair and development of the injury that governs the magnitude of the final injury seen. However, too little is known of the cellular processes that follow irradiation to make further speculation worth while at the present time.

The major need now is for more information about the biochemical lesions. Only when these have been identified will it be possible to check which of the many chemical changes produced both by radiations and by the alkylating agents are important and which are not.

Summary

(1) The biological changes seen after treatment with the alkylating agents and after exposure to ionizing radiations are the end effects of a series of metabolic processes initiated by a chemical change produced in a very few vital macromolecules of the cell.

(2) *In vitro* studies suggest that both types of agent produce their effects by damaging DNA molecules, which are thereby rendered useless to the cell. Proteins and many other molecules present in the cell will also be changed but, since neither radiation nor alkylating agents are likely to react with one protein very much more than with another, these reactions are unlikely to be important because the proportion of any one protein (or enzyme) inactivated will be very small.

(3) In the cell, the nucleoproteins are present in locally high concentrations in which the bifunctional reagents crosslink different DNA molecules together via their phosphate groups, which are accessible even when DNA is combined with protein. This crosslinking process is a particularly effective way of putting DNA out of action, and it explains why polyfunctional agents are so much more effective than monofunctional. The latter can inactivate DNA only after blocking a number of individual phosphate groups, whereas a single cross link produces a profound modification.

(4) With the exception of the phosphate groups, all the reactive centers of DNA such as amino groups are hidden, and this explains why acylating agents and other amino reactors that readily combine with proteins under physiological conditions are not radiomimetic. *In vitro* experiments indicate that by far the simplest way of modifying DNA chemically under physiological conditions is to block the phosphate groups, and it is the ability to do just this that distinguishes radiomimetic alkylating agents from othe agents which, while capable of reacting with cell constituents, are biologically inactive.

(5) DNA is very sensitive to ionizing radiations, which are highly effective in breaking the main polynucleotide chains by direct action; this effect wil predominate in the nucleus because of its high solid content.

(6) Both the alkylating agents and radiation can produce such profound chemical changes in DNA that this is bound to be rendered useless to the cell These reactions are likely to be relatively unselective; all functions of th cell that are directly related to the DNA will therefore be vulnerable. Thi would explain the wide range of effects produced. It is suggested that most if not all, of the differences in biological action between radiation and th

alkylating agents follow from the fact that the latter must diffuse into the cell and that some DNA molecules are therefore more accessible than others to the reagent.

References

1. Bryson, V. 1948. J. Bacteriol. **56**: 423.
2. Chanutin, A. & E. C. Gjessing. 1946. Cancer Research. **6**: 599.
3. Sparrow, A. M. & F. M. Rosenfeld. 1946. Science. **104**: 245.
4. Butler, J. A. V. 1950. Nature. **166**: 18.
5. Boyland, E. 1952. Endeavour. **11**: 87.
6. Bohème, R. & M. Magat. 1951. Compt. rend. **232**: 1104.
7. Jensen, E. V. 1950. Trans. 5th Conf. Biol. Autoxidants. : 59. Josiah Macy, Jr. Foundation. New York, N. Y.
8. Alexander, P. & M. Fox. 1952. Nature. **169**: 572.
9. Stacey, K. A., M. Cobb, S. F. Cousens & P. Alexander. 1958. Ann. N. Y. Acad. Sci. 68(3): 682.
10. Butler, J. A. V., L. A. Gilbert & K. A. Smith. 1950. Nature. **165**: 714.
11. Gray, L. H. 1952. Brit. J. Radiol. **25**: 235.
12. Bacq, Z. M. & P. Alexander. 1955. Fundamentals of Radiobiology. Butterworth. London, England.

13a. Alexander, P. 1952. Nature. **169**: 226.

13b. Alexander, P. 1954. Advances in Cancer Research. **2**: 1.

14. Roberts, J. & G. P. Warwick. 1957. Nature. **179**: 1181.
15. Zamenhof, S., G. Leidy, E. Hahn & H. E. Alexander. 1956. J. Bacteriol. **72**: 1.
16. Taylor, B., J. P. Greenstein & A. E. Hollaender. 1948. Arch. Biochem. **16**: 19.

17a. Alexander, P. 1957. The relative importance of direct and indirect action. *In* Progress in Radiobiology. **2**: 8. Oliver and Boyd. Edinburgh, Scotland.

17b. Alexander, P. 1957. Radiation Research. **6**: 653.

17c. Alexander, P. & A. Charlesby. 1954. Nature. **173**: 578.

18. Lea, D. E., K. M. Smith, B. Holmes & R. Markham. 1944. Parasitology. **36**: 110.

19a. Alexander, P. & K. A. Stacey. 1955. Biochem. J. **60**: 194.

19b. Alexander, P. & K. A. Stacey. 1955. Nature. **176**: 162.

20. Alexander, P. & K. A. Stacey. 1956. Progress in Radiobiology. : 105. Oliver and Boyd. Edinburgh, Scotland.
21. Brown, D. M. & A. R. Todd. 1952. J. Chem. Soc. : 52.

22a. Alexander, P. Unpublished data.

22b. Alexander, P., S. F. Cousens & K. A. Stacey. 1957. Ciba Foundation Symposium on Drug Resistance in Micro-Organisms. : 294. Churchill. London, England.

23. Revell, S. H. 1953. Heredity. Suppl. **6**: 107.
24. Auerbach, C. 1946. Proc. Roy. Soc. Edinburgh. **62**: 211.
25. Koller, P. C. & A. Casarini. 1952. Brit. J. Cancer. **6**: 173.
26. Revell, S. H. 1957. Brit. Empire Cancer Campaign Rept. **34**: 30.

DESIGN OF ALKYLATING AGENTS FOR SELECTIVITY OF ACTION

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Let me assure you from the beginning that even we chemists, engaged i the field of chemotherapy of malignant diseases, accept the fact that th chemicals used thus far in cancer and leukemias do not represent even a semi final solution of this vast problem. However, such acceptance must not b mistaken for a defeatist attitude, leading to a pessimistic treatment of wha is presently available, say, in the field of alkylating agents. Of course, i we knew (1) all about the mechanism of carcinogenesis, (2) every detail o cell and tissue composition, (3) every shade of difference between normal embryonic, regenerating, and abnormal material, and (4) the exact natur of interaction between a drug and the cellular constituent, the change o which produces the greatest therapeutic effect, then we could sit down at desk and construct the most perfect remedies for all forms of malignancy.

As it is, we must give the other investigators time to clear up all the funda mental matters, take up leads (if they offer themselves by chance or design learn as much as possible from the partial successes achieved with the grou of drugs at our disposal at this moment, and try hard to improve on them b diminishing their side effects and by increasing their potency and selectivit of action inside the given limits.

I do not think I need to point out the difficulties when attempting th latter tasks. There are presented in these pages a great number of chemica biological, and clinical observations with a considerable number of alkylatin agents. But how many of the drugs mentioned have been compared unde identical conditions if not clinically, at least experimentally? Howeve if we accept a basis of relatively crude comparisons of their action on macr molecules—including transforming principles (Stacey and Alexander *et al.*, 682)—on experimental tumors (Stock and Sugiura, p. 853, Creech, p. 868 with regard to carcinogenesis (Walpole, p. 750), on cytological propertie (Koller, p. 783), on blood-forming elements (Elson, p. 826), on pharmacolog cal behavior (Sternberg *et al.*, p. 811), on mutagenesis (Auerbach, p. 73 Fahmy and Fahmy, p. 736) and, last but not least, on human patients, I sha present what information there is and, in some respects, what there could with regard to the selectivity of alkylating agents.

First, a few general remarks. The existence of differences in the desirab and undesirable activities of these drugs must derive from circumstanc similar to those controlling the effectiveness of other chemotherapeutics ar symptomatic drugs. May I stress the fact that, for the latter, the proble of Structure Activity Relationship (SAR), expressed by Crum-Brown ar Fraser[1] in 1869 in the form of a pseudo-equation $\phi = f(C_D)$, which could extended to $\phi = f(C_D) \cdot (C_R)$, has exercised and is exercising the minds many people. Ing[2] has distinguished between pharmacodynamically acti groups in the molecules and their carrier parts; thus, the alkylating agents can be considered to be composed of "warheads" (monofunctional or polyfunctional) and, in many instances, of carriers consisting of rings, radicals, and functional portions. Under biological conditions, from the moment of administration (FIGURE 1) both parts can influence (1) a favorable conditioning of drug effects—that is, the ease of transport and distribution (discussed by Smith), the concentration and/or selective activation at the site of relevant receptor centers [Alexander and Stacey's (page 1225) phosphates in nucleoproteins or Roberts and Warwick's[3] SH groups?], and the absorption and final desirable interaction with these centers; and (2) the unfavorable conditioning—that is, high rate of decomposition (mainly by water), reaction with irrelevant centers other than water, detoxication and excretion, and reversal of reaction with relevant centers. From Price (p. 663) and from Ross (p. 669) we have heard that the probability of reaction with relevant centers varies with the alkylating agent, which can follow a monomolecular

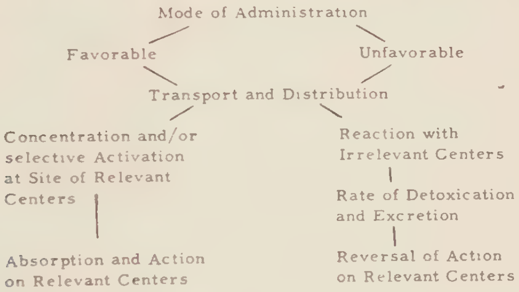
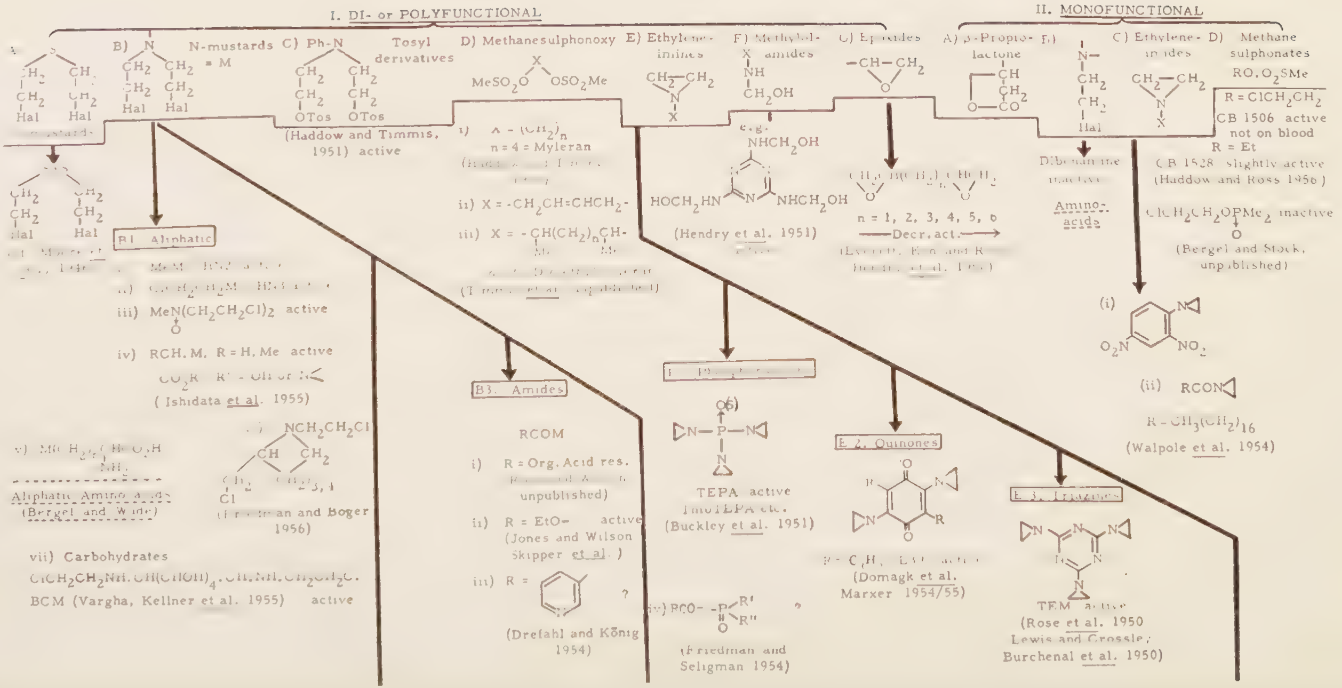

Mode of Administration
Favorable
Unfavorable
Transport and Distribution
Concentration and/or selective Activation at Site of Relevant Centers
Absorption and Action on Relevant Centers
Reaction with Irrelevant Centers
Rate of Detoxication and Excretion
Reversal of Action on Relevant Centers

FIGURE 1

S_N1, a bimolecular S_N2, or a mixed type of reaction mechanism. Ross speaks of the rates of hydrolysis or reaction with thiosulfate as an approximation of chemical reactivity of alkylating agents, and considers the reactivity of the active groups of these agents in terms of competition factors and concentration, using the formula $F \times f \times c$. Some S_N2 reactors have very high differential F values for RS ions and NH_2 as against acid ions; others, such as methanesulfonoxy alkanes, have small differentials. Consequently, the latter are less likely to be "mopped up" by irrelevant centers. According to Ross, the same consideration applies to S_N1 reactors, because the fixed rate of ionization (see Cromwell's discussion, p. 721), rather similar to the fixed rate of disintegration of radioactive materials, gives them a better chance of arriving unscathed at the relevant sites. Of course, the situation is far too complex to be squeezed into a few mathematical expressions. The questions as to whether the diminution of unfavorable conditioning or the increase of favorable conditioning will lead to greater selectivity, and whether the difference between the biological effects of, say, chlorambucil and Myleran, as pointed out by Elson,[1] is sufficiently large to bear this out, cannot be answered yet with absolute certainty.

While I must re-emphasize the uncertain basis of comparison, I should like to select from FIGURE 2 a number of relatively strongly supported points


I. DI- or POLYFUNCTIONAL
II. MONOFUNCTIONAL
B) N N-mustards = M
Hal Hal
C) Ph-N Tosyl derivatives
OTos OTos
(Haddow and Timmis, 1951) active
D) Methanesulphonoxy
MeSO2O OSO2Me
i) n = 4 = Myleran
ii) X = -CH2CH=CHCH2-
iii) X = -CH(CH2)nCH-
E) Ethylene-imines
F) Methylol-amides
e.g.
NHCH2OH
HOCH2HN NHCH2OH
(Hendry et al. 1951)
G) Epoxides
n = 1, 2, 3, 4, 5, 6
Decr. act.
A) β-Propio-lactone
B)
Hal
Dibenamine inactive
Amino-acids
C) Ethylene-imides
D) Methane sulphonates
RO.O2SMe
R = ClCH2CH2
CB 1506 active not on blood
R = Et
CB 1528 slightly active
(Haddow and Ross 1956)
ClCH2CH2OPMe2 inactive
(Bergel and Stock, unpublished)
(i)
O2N NO2
(ii) RCON
(Walpole et al. 1954)
B1 Aliphatic
iii) MeN(CH2CH2Cl)2 active
iv) RCH.M, R = H, Me active
(Ishidata et al. 1955)
NCH2CH2Cl
CH CH2
Cl
an and Boger 1956)
Aliphatic Amino acids
(Bergel and Wade)
vii) Carbohydrates
BCM (Vargha, Kellner et al. 1955) active
B3. Amides
RCOM
i) R = Org. Acid res.
unpublished)
ii) R = EtO- active
(Jones and Wilson
Skipper et al.)
iii) R =
?
(Drefahl and König 1954)
TEPA active
ThioTEPA etc.
(Buckley et al. 1951)
iv) RCO-
(Friedman and Seligman 1954)
E 2. Quinones
(Domagk et al.
Marxer 1954/55)
E 3. Triazines
TEM active
(Rose et al. 1950
Lewis and Crossley;
Burchenal et al. 1950)

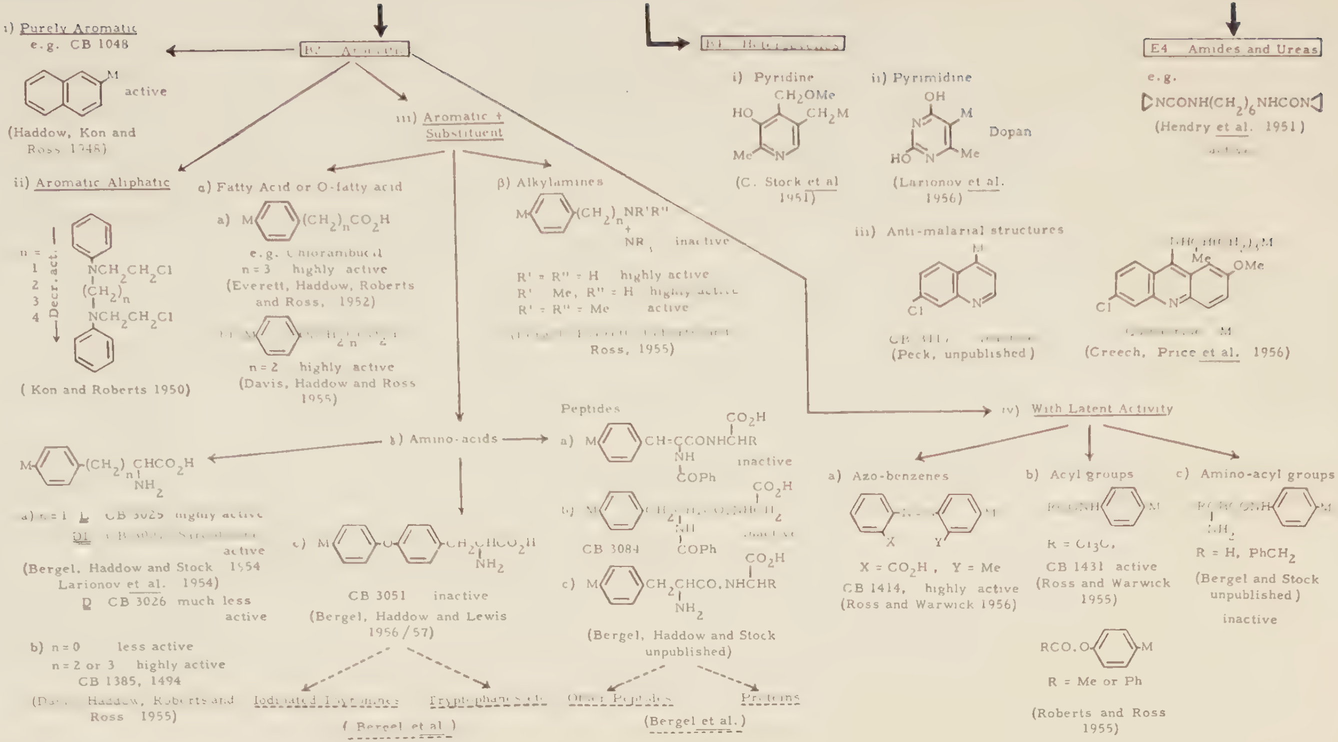


FIGURE 2. Design of alkylating agents for selectivity of action.

that will, I hope, illustrate some of the possibilities of a crude design for selectivity of action in this field.

Difunctional sulfur mustards (I A) have been studied extensively from a reaction point of view and have been reviewed with other alkylating agents by Ross,[5] but have received only very restricted attention concerning variation of structure (see Petrakis *et al.*, p. 1151). I shall return to the monofunctional or hemisulfur mustards shortly.

Leaving for later consideration the nitrogen mustard derivatives (I B), the largest and most varied group, we arrive via the tosyl derivative (C) at the methanesulfonoxy alkanes (D), with 1,4-dimethane sulfonoxybutane (Myleran) and 1,4-dimethane sulfonoxy-1,4-dimethylbutane (dimethyl Myleran).[6] While claims for the specificity of action of Myleran (see also 1,9-dimethane sulfonoxynonane Nonane tested by Sykes *et al.*[7]) on levels of myeloid forms of leukocytes generally remain undisputed, dimethyl Myleran has been mentioned in this monograph by Bierman *et al.* (p. 1211) as having unexpected effects. Whether this change from a series of primary glycols to secondary ones, with increased hydrolysis rates and altered differential reactivity toward nucleophilic centers, represents a true move toward selectivity must be left to the outcome of future experimental and clinical studies.

Replacement of a sulfonoxy radical by a chlorine atom (II D) as in β-chloroethyl methanesulfonate (CB 1506), tentatively quoted as monofunctional, prepared by Ross and investigated by Haddow,[8] Elson, Galton, and by Fahmy and Fahmy,[9] leads to a material that has lost the effects on the bone marrow, but seemingly has gained special mutagenic properties. Roberts has investigated the related ethyl methanesulfonate (CB 1528), which *S*-ethylates cysteine in the rat. Whether CB 1506 chloroethylates SH compounds such as cysteine and exerts its biological action indirectly via a metabolite is still an open question. In this connection it is an interesting point that the hemisulfur mustard

$$Cl{\cdot}CH_2{\cdot}CH_2{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}OH$$

mentioned before, is somewhat related to *S*-chloroethyl cysteine

$$Cl{\cdot}CH_2{\cdot}CH_2{\cdot}S{\cdot}CH_2{\cdot}CH(NH_2)CO_2H$$

(CB 1592), which is being tested. Seligman *et al.* have reported earlier[10] and in this monograph (p. 1181) on their clinical experience with the former compound, which also has no effect on blood-forming elements. I should mention that the last compound in group II D in FIGURE 2, 2-chloroethyldimethy phosphinate, proved to have hardly any activity against the Walker rat carcinoma; this negative result, as any other, is just as much an illustration o SAR as are quantitative differences among positive results.

We now come to the ethylenimines (I E). In this group a number o different carriers have been used: phosphoric and thiophosphoric acids including morpholino derivatives (for example, oxapentamethylenediethylene thiophosphoramide OPSPA investigated by Curreri[11] and by McIve *et al.*, p. 1183, but not shown in FIGURE 2); quinones (see Domagk, p. 1197) triazines; and aliphatic dicarboxylic, dicarbamic, and disulfonic acids [fo example, 1,3-bis(ethyleniminosulfonyl)propane, BEP[12,13]. This should give one the opportunity to assess the importance of the carrier part of the molecule for selectivity of action in this group. In spite of various claims made for differences in clinical usefulness (see Wright *et al.*, p. 937) there appears to be available at this moment no definite evidence to support these claims.[7] It is a great pity that ethylenimino radicals, which are always combined with acidic or near-acidic carriers and which at biological *p*H may follow a S_N2-type reaction mechanism similar to that of the epoxides, have not yet been combined with amino acids or with ring systems carrying acidic or basic side chains. One very important fact has emerged from the work by Walpole *et al.*[14] on monofunctional ethylenimine derivatives of fatty acids; namely, that they show carcinogenic but little if any antitumor activity. Whether this separation of the two effects, which usually go together (Haddow effect), can be achieved with other monofunctional agents remains to be seen.

In a brief review I can do no more than mention the methylolamides (I F)[15] and the epoxides (I G); the latter, according to Stacey, differ in their *in vitro* effects on macromolecules from nitrogen mustards and which, according to Westergaard *et al.*,[16] have given interesting results in the mutagenesis of *Neurospora*.

I should like to concentrate now on the nitrogen mustard group and its derivatives (I B), with which my colleagues and I have a more extensive experience than with any of the other alkylating agents except, of course, the methanesulfonoxy derivatives. The main subdivisions, as given in FIGURE 2, are: (1) aliphatic; (2) aromatic; (3) amides; and (4) heterocyclic derivatives. There is not very much support from the clinical side for the theory that the nitrogen mustard group confers a specific action on the structure carrying it, when such action is compared with those of other alkylating groups. May I point out to our clinical friends that, apart from a different action mechanism followed by the aromatic series, first, there is an undisputed hematological difference between nitrogen mustards and methane sulfonoxy derivatives, if one remains inside a range of low doses with the latter (see Galton *et al.*, p. 967). Second, as mentioned before with the ethylenimines, the assessment of finer degrees of distinction is made impossible by the absence of comparable structures substituted with the nitrogen mustard group or with ethylenimino radicals. I feel that it is too early to arrive at such a statement, which ignores the importance of quantitative differences, in contrast to qualitative ones. Moreover, the presence or absence of side effects, the rate of production of drug resistance—all these and many more properties must be taken into account before coming to a final conclusion.

Experimentally, there is no doubt that a considerable difference exists between the aromatic and aliphatic derivatives. Among the latter (B 1), BCM, the mannitol mustard of Vargha *et al.*,[17] of Kellner, and of Sellei and Eckhardt (p. 1164) has aroused our interest. As a glance into the future, I may mention that R. Wade and I are at present studying the synthesis of chloroethylamino derivatives corresponding to lysine and ornithine and carrying one or two chloroethyl residues on the ω-amino group.

Now to consider the aromatic series (B 2 iii): the introduction of acidic side chains gave chlorambucil (Ross *et al.*[18]); of basic side chains[19] (with the exception of quaternary compounds) gave substances that show an extraordinary compensatory neutrophilic effect (Elson, p. 826); and the introduction of an amino acid gave Melphalan (phenylalanine nitrogen mustard, CB 3025).[20a, b] The latter is the L-isomer corresponding to Larionov's[21] racemic sarcolysin, and, from the point of view of dose level, it is still the most active substance against the Walker tumor and a Russian rat sarcoma. Apart from HN2, it is also the most effective compound in bringing about an inhibition of the *Hemophilus influenzae* transforming principle of Zamenhof and his associates.[22] In addition, Fahmy and Fahmy[23] claim special mutagenic properties for Melphalan in *Drosophila*. While higher homologues of CB 3025, namely CB 1385 and CB 1494,[24] are apparently not very much less effective in the experimental conditions just mentioned, the amino acid side chain alone is no guarantee for high activity, as demonstrated with the inactive CB 3051.[25] The same tenet applies to some *N*-benzoyl dehydropeptides and peptides. However, my colleagues and I are convinced that the "amino acid idea" as such is sound, and we are therefore seeking to achieve the synthesis of nitrogen-mustard-carrying NH_2-free peptides and even proteins and of iodinated thyronine and of tryptophan derivatives.

Ross (p. 669) mentioned briefly the concept of mustard derivatives with latent activity (B 2 iv); that is to say, consisting of a structure that will diminish the reactivity of the nitrogen mustard group as an alkylating group unless the molecule breaks down under biological conditions to an activated moiety. The azobenzene series of Ross and Warwick,[26] and the acyl mustard anilines and phenols have illustrated the soundness of this working hypothesis,[27] which is also followed by Friedman and Seligman[28] in a different direction. Strangely enough, α-amino acyl derivatives have shown little activity. On the other hand, di-(chloroethyl) amides (B 3) are gaining in interest in view of the biological activity of mustard derivatives of urethan (B 3 ii)[29] in which the mustard group shows very low chemical reactivity.

There remain a number of heterocyclic derivatives (B 4) developed by various laboratories.[30-32] In this publication Creech (p. 868) and Jones *et. al.* (p. 1133) have discussed the activities of the antimalarial analogues of the nitrogen mustards. Another recent addition to this division is the benzimidazole derivative mentioned by Hirschberg *et al.* (p. 888).

I fear I may have committed many sins of omission in this summary. Some of my readers may still feel that the case for even quantitative differences among alkylating agents is "not proved." Nevertheless, I hope I have made a successful plea for the existence of these differences. In view of the incomplete state in which we find this chapter of cancer chemotherapy, I can only express the sincere hope that one day the fabric of concepts and results in this field will not be woven of a plethora of woof with a paucity of warp, but will be strong and solid. Of course, by then the "big break" may have come, from a different direction, changing the "presulfonamide" days, or the days of alkylating agents, into the golden days of specific chemical treatment of all forms of malignant diseases.

References

1. Crum-Brown, A. & T. R. Fraser. 1868/69. Trans. Roy. Soc. Edinburgh. **25**: 151.

2. Ing, H. R. 1943. Trans. Faraday Soc. **39**: 372.

3. Roberts, J. J. & G. P. Warwick. 1957. Nature. **179**: 1181.

4. Elson, L. A. 1954. Symposium of Radiobiology. : 235. Z. M. Bacq & P. Alexander, Eds. Butterworth. London, England.

5. Ross, W. C. J. 1953. Advances in Cancer Research. : 397. J. P. Greenstein & A. Haddow, Eds.

6. Haddow, A. & G. M. Timmis. 1951. Acta Unio Intern. contra Cancrum. **7**: 469.

7. Sykes, P. M., F. S. Philips & D. A. Karnofsky. 1956. Med. Clin. N. Am. **40**: 837.

8. Haddow, A. & W. C. J. Ross. 1956. Nature. **177**: 995.

9. Fahmy, O. G. & M. J. Fahmy. 1956. Nature. **177**: 996.

10. Seligman, A. M., A. Rutenberg, L. Persky & O. M. Friedman. 1952. Cancer. **5**: 354.

11. Curreri, A. R. & F. R. Russo. 1955. Proc. Am. Assoc. Cancer Research. **2**: 11.

12. Hendry, J. A., F. R. Homer, F. L. Rose & A. L. Walpole. 1951. Brit. J. Pharmacol. **6**: 357.

13. Paterson, E. & P. B. Kunkler. 1954. Ciba Foundation Symposium on Leukaemia Research. : 231. J. & A. Churchill. London, England.

14. Walpole, A. L., D. C. Roberts, F. L. Rose, J. A. Hendry & R. F. Homer. 1954. Brit. J. Pharmacol. **9**: 306.

15. Hendry, J. A., F. L. Rose & A. L. Walpole. 1951. Brit. J. Pharmacol. **6**: 201.

16. Jensen, K. A., I. Kirk, G. Kølmark & M. Westergaard. 1951. Cold Spring Harbor Symposia Quant. Biol. **16**: 245.

17. Vargha, L., L. Toldy, Ö. Fehér & S. Lendvai. 1957. J. Chem. Soc. : 805.

18. Everett, J. L., J. J. Roberts & W. C. J. Ross. 1953. J. Chem. Soc. : 2386.

19. Bergel, F., J. L. Everett, J. J. Roberts & W. C. J. Ross. 1955. J. Chem. Soc. : 3835.

20a. Bergel, F. & J. A. Stock. 1954. J. Chem. Soc. : 2409.

20b. Bergel, F. & J. A. Stock. 1953. Brit. Empire Cancer Campaign Rept. **31**: 6.

21. Larionov, L. F., A. S. Khokhlov, E. N. Shkodinskaja, O. S. Vasina, V. I. Troosheikina & M. A. Novikova. 1955. Lancet. **2**: 169.

22. Zamenhof, S., G. Leidy, E. Hahn & H. E. Alexander. 1956. J. Bacteriol. **76**: 1.

23. Fahmy, O. G. & M. J. Fahmy. 1956. J. Genet. **54**: 146.

24. Davis, W., J. J. Roberts & W. C. J. Ross. 1955. J. Chem. Soc. : 890.

25. Bergel, F. & G. E. Lewis. 1957. J. Chem. Soc. : 1816.

26. Ross, W. C. J. & G. P. Warwick. 1956. J. Chem. Soc. : 1364.

27. Ross, W. C. J., G. P. Warwick & J. J. Roberts. 1955. J. Chem. Soc. : 3110.

28. Friedman, O. M. & A. M. Seligman. 1954. J. Am. Chem. Soc. **76**: 655.

29. Skipper, H. E., C. E. Bryan, W. H. Riser, M. Welty & A. Stelzenmuller. 1948. J. Natl. Cancer Inst. **9**: 77.

30. Stock, C. C., S. Buckley, K. Suguira & C. P. Rhoads. 1951. Cancer Research. **11**: 432.

31. Larionov, L. F. 1956. Brit. J. Cancer. **10**: 26.

32. Jones, R., H. J. Creech, C. C. Price, A. K. Sen, R. M. Peck, R. F. Hankwitz, D. McKenzie & W. F. Dunning. 1956. Proc. Am. Assoc. Cancer Research. **2**: 122.

PROCEDURES RECOMMENDED FOR THE CLINICAL TRIAL OF ALKYLATING AGENTS

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The problem of how to judge the clinical effectiveness of alkylating agents is clearly part of the more general problem of how to study the therapy of all neoplastic disease. More specifically, the difficulties that confront us are those concerned with the clinical trial of drugs in patients with the "solid" or nonleukemic tumors. A group of investigators for whom I am spokesman has been attempting a study of therapeutic trials with such tumors during the past year. These investigators include Holland, Chalmers, Lasagna, Jones, Shnider, Brindley, and Tuohy. We should be the last to say that we have found satisfactory procedures to recommend, as perhaps the title of this paper suggests, but I shall try to describe the present status of our self-education.

The way in which drugs are studied or, as some would say, the experimental design of a therapeutic trial is determined in large measure by what the investigator wishes to know. The investigator may be interested only in discovering drugs that will produce dramatic regression in disseminate cancer. For this purpose the simplest plan of study suffices, and this plan is one with which we are all familiar. Its essence may perhaps be summarized in a remark heard occasionally: "When the miracle drug appears, I won't need a statistician to tell me that it is effective." In this instance the investigator wishes to know only about the markedly positive drugs; he is content with this qualitative determination: either the drug is strongly positive or it is not. This is a perfectly valid approach and may well be th means by which truly active drugs are found. Certainly in diseases othe than cancer, this treasure-hunting type of approach has occasionally paid of handsomely. However, as the years pass and the curative drug for cance does not appear, the question arises as to whether this is the only informatio desired from a clinical trial. Or, to rephrase the problem, would cance chemotherapy move ahead more rapidly if, in trials of antitumor drugs, on asked and attempted to answer a different set of questions?

After examining the methods of other successful chemotherapy programs our group decided that there were other questions to be asked. For example

The biologist What animal tumors are best for predicting antitumo activity in patients?

The biochemist Which theory of growth inhibition is valid in man?

The organic chemist Which analogues of known active drugs posses increased activity?

The antibiotic manufacturer When active antibiotic beers are subjecte to fractionation, in which fraction is there greatest activity?

In my opinion, the answers to these and similar questions are vital to th advance of cancer chemotherapy, just as they were vital to the successfu prosecution of chemotherapy programs in infectious diseases. They are vital because the search for curative drugs is stopped at the treasure-hunting phase until the quantitative data of the basic scientist can be validated in man by comparison with equally precise data. The acquisition of such data has been possible in other diseases of man, and it would seem logical that, by application of the same principles, precise data could be secured in the study of cancer therapy. If the investigator is not content with treasure hunting but, in addition, wants to know in a quantitative way the correlation between drug response in experimental systems and drug response in patients with tumors, then a different type of therapeutic trial is required. It is my purpose to review some of the difficulties encountered in such quantitative drug trials.

Most of the discussion about therapeutic trials can be reduced to two main questions: (1) What is to be measured? (2) How are these measurements to be used—(a) to discover if a new drug is active, or (b) to compare the activities of two active drugs?

What Is To Be Measured?

The response of the leukemias in man has been a most sensitive index of drug activity. In judging the effect of drugs on solid tumors, however, the end point is much less satisfactory. An analysis of why it is that the response in the leukemia patient is so readily measured makes it apparent that these criteria of tumor inhibition in the leukemias are: (1) objective; (2) capable of supplying an answer in a reasonable time; (3) a direct reflection of what is happening to the tumor; and (4) involve measurement of a commonly found attribute of tumors.

The success enjoyed in obtaining quantitative data from the study of leukemia by such criteria[1] has led us to feel that criteria of tumor inhibition of solid tumors should have the same characteristics. Gellhorn's review (pp. 1254) made it clear why it is axiomatic that criteria of effect must be objective. However, criteria may be objective and yet not very useful for clinical trials. The emphasis of objectivity alone, without reference to items 2, 3, and 4, is the reason for the poor end point in drug trials in solid tumors. For example, to obtain useful data the answers must be obtainable in a reasonably short time. Thus, the five-year cure rate, although objective and a direct measure, is not useful in the kind of study I am discussing. If a decision on drug activity requires five years, or even one year, the chemist and the biologist cannot be furnished information on the validation of their hypotheses in man rapidly enough to plan their next step.

Other objective measures of tumor effect are shown in TABLE 1. These cannot be used by themselves as direct measures of effectiveness, since they are nonspecific and may undergo increases or decreases for many reasons other than tumor growth. Under highly specialized circumstances some of these measures may be narrowly useful.

In another category are substances shown in TABLE 2. These materials are chemical products of tumors, can be measured, and give at least a rough index of the amount of tumor present. Three of these tumors are very rare while, with cancer of the prostate, the test is positive in less than half the cases. Except perhaps for acid phosphatase, these chemical measures of tumor growth are not sufficiently common attributes to justify wide application.

TABLE 1
NONSPECIFIC MEASURES OF TUMOR GROWTH

Fever
Anemia
Weight loss
Specific organ function
Calcium balance
Serum enzymes
Serum uric acid
Serum polysaccharides
Serum proteins

It was our decision that none of the above are generally useful measures of chemotherapeutic effect. Only two parameters remain the estimation of tumor mass by direct observation, and survival time. At the present time these modalities are, in my opinion, the only direct and objective measures of chemotherapeutic effect that can be widely applied to the study of the therapy of solid tumors.

TABLE 2
METABOLITES PRODUCED BY TUMORS

Prostate—acid phosphatase
Myeloma—abnormal proteins
Carcinoma adrenal cortex—steroids
Carcinoid—5-hydroxy indole acetic acid
Choriocarcinoma—gonadotrophins

Estimation of tumor mass. Total tumor mass can be measured both by the size of the tumor and by the number of metastases. In regard to measurement of tumor size, the primary tumor is less satisfactory than the metastases. By the time patients are presented for chemotherapy the primary is often large and of indefinite extent, and has a poor blood supply, many areas of necrosis, and much surrounding fibrosis and inflammation. Changes due to an effective agent might therefore be expected to be slow and to require prolonged drug administration. This is true in similar granulomatous masses resulting from infection For example, in subacute bacterial endocarditis[2] the effect of penicillin was missed at first because of slow response.

A second point to be emphasized in the measurement of tumor size is that only those patients in whom the tumor mass can be measured directly are suitable candidates for this type of study. Quantitative data cannot be obtained if the tumor mass is not accessible, as is the case in the retroperitoneal area, abdominal cavity, skull, or gastrointestinal tract. Primary pulmonary tumors are often quite difficult to measure repetitively because of associated atelectasis, obstructive pneumonia, and pleural fluid. Thus, if tumor size is to be the end point, only patients with easily measurable disease should be studied. The most useful of these are cutaneous and subcutaneous metastases, peripheral lymph node disease, and osseous and pulmonary metastases. In practice, the tumor masses are measured with a pair of calipers about twice a week. Least variable results are obtained if al measurements are made by the same observer, who is kept unaware of which drug the patient is receiving. Some of the curves on tumor growth obtained by such methods are shown in FIGURES 1 and 2. These methods have been developed by Clyde Brindley of the National Cancer Institute, and are presented with his kind permission.

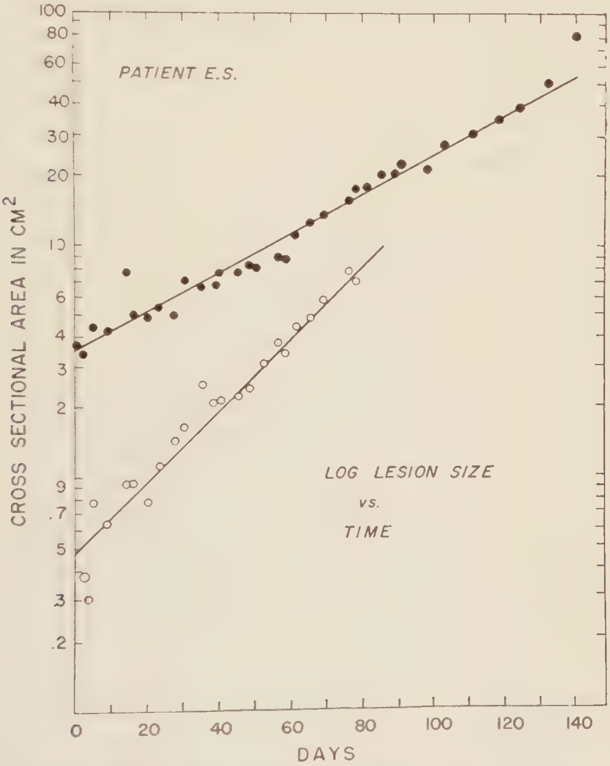

FIGURE 1. Growth rates of two metastatic lesions from a patient with malignant melanoma.

By the plotting of such measurements, it is possible to fit a line to the points and obtain an expression for rates of growth of the tumors. The same can be done for the number of metastases appearing (FIGURE 2). A positive effect from therapy consists of a statistically significant change in the slopes of the lines for the growth rates of the total tumor mass It would be foolish to hope that the growth rates of all lesions would fall so close to a straight line and, indeed, there are a number of distressing variations. However, thus far it has been our experience that fitting such lines to measures of tumor size and tumor number is quite feasible and useful. We believe this to be an effective method for quantifying the behavior of the total tumor mass in a patient, and for relating the changes in growth rate to therapy.

Survival times. In some diseases such as cancer of the lung, accurate measurement of tumor mass is often impossible. In these instances the best quantitative index of therapy is survival time. As emphasized by Merrell[3] and others,[4,5] the use of the Life Table analysis of survival times provides us with a highly satisfactory way of judging the effects of therapy. This results in an objective, direct index of tumor growth that, in many tumors, can give an answer in a relatively short time.

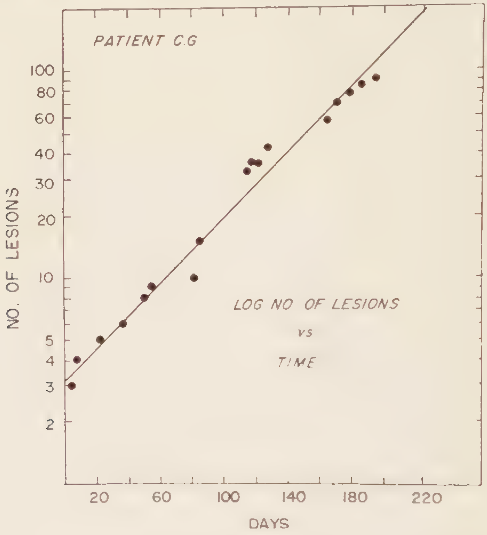

FIGURE 2. Rate of appearance of new metastatic lesions in a patient with malignant melanoma.

TABLE 3

EFFECT OF CB 1348 ON LYMPHOMA. PER CENT OF "EXCELLENT RESULTS"

Study Number	Hodgkin's disease	Chronic lymphocytic leukemia	Lymphosarcoma
1	0	0	0
2	0	11	7
3	11	45	35
4	33	75	33
5	66	50	20

As a final point on indices of tumor growth, I should like to stress the importance of standard criteria. Some of the results of the investigations reported in this monograph are shown in TABLE 3. Note the wide variation in the following definitions of "Excellent Results" employed by different investigators: (1) subsidence of all objective and subjective disease with return to full activity for 6 months or more; (2) regression of all disease for 6 months or more; (3) regression of all apparent disease, but without reference to duration of regression; (4) regression of one or more nodules without evidence of progression elsewhere; and (5) regression of any single tumor lesion. The reason for differences in results is apparent from the wide range of these criteria used for the definition of a positive effect.

In addition, there exists the problem of drug dosage. The authors included in the section on clinical results used different amounts of drug, different routes of administration, different dosage schedules, and different durations of administration. In comparing one alkylating agent with another, one author used one drug for several months and the other for a few days, and then drew conclusions about comparative efficacy. If those interested in chemotherapy would agree arbitrarily on the criteria for positive effect, and for all other conditions for the study, then all would be talking the same language, and one of the major sources of variation would be eliminated. Those working with leukemia have demonstrated the usefulness of standard criteria;[1] I should like to suggest that solid tumor chemotherapy would similarly benefit.

How Are These Measures To Be Used?

In the trial of therapy in disease states it is assumed that the physician knows the disease so well that he can predict what its course will be, and therefore can tell quickly the deviations from this predicted course brought about by the therapy. Experience with therapy in many diseases has shown that this is not a valid assumption. The drug therapy of malaria,[6] of pulmonary tuberculosis,[7, 8] and the rheumatic diseases,[8] to mention but a few, has been subjected to intense scrutiny by groups in the United States and in Great Britain, and the conclusions of all have been the same physicians cannot predict the course of even the common diseases with sufficient accuracy to interpret the effect of therapy. The therapy of cancer is no exception and, in my opinion, is much worse because cancer is so many diseases. Improvements in general medical knowledge have altered the outlook of patients with cancer so that the natural history of cancer today is not the natural history of cancer yesterday, and the base lines of yesterday often are not helpful. To many of us it seems impossible to give a small number of cancer patients a drug and determine what the drug has done by comparison with some vague and indeterminate expected behavior. In all major therapeutic trials conducted by the Medical Research Council,[8, 9] the Veterans Administration,[7] and others,[10] this problem of the base line has been solved by running concurrent study groups, where half the patients receive the best known care and the other half receive the same care plus the new drug to be studied. From our experience to date, this is essential to the study of the therapy of all cancer, but especially of chemotherapy. The principles for the conduct of such trials have been repeated many times elsewhere,[7, 9, 11, 12] and I shall present the generally accepted conclusions without making any attempt to defend them (TABLE 4).

For direct comparison of one drug with another, this procedure is essential.

I believe also that adherence to these principles in even the earliest exploration of new drugs for therapeutic activity will give a more precise answer in a shorter time and with fewer patients. One of the techniques that many in this field are discussing is the application of sequential analysis theory in the early exploration of new drugs for effectiveness,[13] and studies are in progress in several clinics using sequential designs.[14] The successful use of sequential designs presupposes a reasonably predictable behavior of a few patients with tumors. It is my feeling at the present time that sequential analysis in cancer chemotherapy would require the use of concurrent pairs.

TABLE 4

PRINCIPLES OF A THERAPEUTIC CLINICAL TRIAL*

(1) The cooperation of individuals with appropriate skills at all levels of planning and execution.
(2) Randomization.
(3) Blind determination of effect.
(4) Statistical treatment of data.
(5) Cautious generalization.

* Reprinted from Lasagna[11] with permission of the author and of the *Journal of Chronic Diseases.*

In summary, it is obvious that our self-education has not led us beyond the kindergarten stage and, as one does in the kindergarten, I have resorted to oversimplification. However, I present these conclusions: There are two general approaches to the problem of drug trials in human cancer— the one governed by the desire to uncover highly active agents, the other governed by the desire to gain quantitative data in man to compare with the quantitative data in the laboratory model. The former approach is based on the hope of suddenly coming upon the answer in full bloom; the latter is based on the thought that all successes and all failures in man are important and require correlation with the successes and failures of the laboratory models, so that ultimately a complete picture of neoplastic response to drugs can be drawn. It is impossible to say that one approach is right and the other wrong or that one will be more successful than the other. Both have been useful in other diseases. In general, the easier the answer, the more useful the treasure-hunting approach; the more obscure the problem, the more useful the systematic gathering of precise data. In the present state of our knowledge, neither approach should be employed to the exclusion of the other. Many of the clinical studies of alkylating agents have been of the treasure-hunting type and, as evidenced in the many fine papers of this monograph, a great deal has been learned. On the other hand, in addition to taking part in the treasure hunt, we as clinicians should rise to the challenge presented to us elsewhere in this monograph by Walpole, who emphasized the need for attempting to attain in clinical trials data of objectivity and precision comparable to those of the laboratory scientist.

References

1. BISEL, H. F. 1956. Blood. **11:** 676.
2. RICHARDS, A. N. 1942. J. Am. Med. Assoc. **122:** 235.
3. MERRELL, M. & L. E. SHULMAN. 1955. J. Chronic Diseases. **1:** 12.

4. LEES, T. W. 1956. Acta Radiol. Suppl. **132.**
5. SMETANA, H. F. & B. M. COHEN. 1956. Blood. **11**: 211.
6. SHANNON, J. A. & D. P. EARLE. 1945. Bull. N. Y. Acad. Med. 2nd Series. **21**: 467.
7. TUCKER, W. B. 1953. Trans. 12th Conf. Chemotherapy of Tuberculosis. Veterans Administration, Army and Navy. **12**: 13.
8. THE JOINT COMMITTEE OF THE MEDICAL RESEARCH COUNCIL AND THE NUFFIELD FOUNDATION. 1954. Brit. Med. J. **1**: 1223.
9. MEDICAL RESEARCH COUNCIL. 1948. Brit. Med. J. **2**: 769.
10. DOULL, J. A. 1954. Intern. J. Leprosy. **22**: 377.
11. LASAGNA, L. 1955. J. Chronic Diseases. **1**: 353.
12. ZUBROD, C. G. 1957. Proceedings of 3rd National Cancer Conference. : 443. Lippincott. Philadelphia, Pa.
13. ARMITAGE, P. 1954. Quart. J. Med. **23**: 255.
14. WOLF, S. 1955. Trans. 14th Conf. Chemotherapy of Tuberculosis. Veterans Administration, Army and Navy. **14**: 278.

THE STATUS OF ALKYLATING AGENTS IN CLINICAL CANCER CHEMOTHERAPY

By Alfred Gellhorn

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It is the purpose of this paper to summarize the clinical studies presented in Part III. Although throughout this monograph much emphasis has been placed on the importance of objective appraisals, I must admit that this summary necessarily will be colored by subjective factors. It constitutes the reactions of one individual to the evidence presented and, therefore, the conclusions drawn do not necessarily reflect a consensus of the contributing investigators.

The Place of the "Conventional Five" in Clinical Therapy (Nitrogen Mustard, Triethylene Melamine, Triethylene Thiophosphoramide, Chlorambucil, and Myleran)

There appears to be agreement on the indications for systemic therapy with the drugs. The susceptible tumors included: the malignant lymphomas (Hodgkin's disease and the lymphosarcomas) when the neoplasms are generalized and symptomatic; the malignant lymphomas when they are localized and producing pressure on vital structures, such as on the great vessels and trachea in the mediastinum or on the spinal cord; chronic myeloid leukemia as soon as diagnosis has been established; chronic lymphoid leukemia when symptoms due to anemia or tumor infiltration appear; ovarian carcinoma when the disease can no longer be controlled locally by surgery or radiotherapy.

There were other diseases wherein systemic therapy with the alkylating agents could be considered, but the response was less certain. Bronchogenic carcinoma was noted to respond, but objective improvement lasted only for brief periods, and attempts to prolong the remission by larger dosage of the drugs led to toxicity. Preliminary studies of chemotherapy of chronic lymphoid leukemia from the time of diagnosis were reported, but the period of follow-up was too brief to permit any conclusion on the merit of this regimen as compared with the usual procedure. Chorioepithelioma was noted to respond with sufficient regularity so that alkylating drug therapy should certainly be employed at some time in the natural history of this rare tumor. Carcinoma of the breast treated by systemic administration of the alkylating agents was reported, and the results did not appear promising to me despite the favorable conclusion reached by one of the investigators. It is fortunate that a cooperative study by one of the large clinical groups supported by the National Cancer Chemotherapy Service Center in Washington has this issue under definitive study. An authoritative answer can be anticipated in the near future.

There was a third group of tumor types in which a favorable response was noted occasionally—a rare gastrointestinal cancer, multiple myeloma, malignant melanoma, and so on. These sporadic responses are difficult to assess and place a great responsibility on the clinician who has observed them. Should each "occasional" be reported? It is felt that the investigator must decide whether or not he wishes to urge his colleagues to use chemotherapy routinely in the management of the particular tumor in question. If the reproducibility of favorable response is so low as to contraindicate such advice, then it is probably wiser to consider the "occasional" success as an internal problem for further investigation.

The Specificity of Antitumor Effect in Man Shown by the "Conventional Five"

Production of evidence on this subject is one of the major objectives of this publication. Surprisingly little data are presented to allow a firm conclusion. The suggestion that Myleran is a specific myelo-proliferative depressant appears to have been countered by the demonstration that this drug can produce objective decrease in the size of bronchogenic cancer comparable to that of nitrogen mustard and can produce remissions in the malignant lymphomas as well. Certainly it was convincingly demonstrated that triethylene melamine can be as effective as Myleran in chronic myeloid leukemia. Recognizing, then, that the amount of evidence was meager, that which was available failed to show qualitative differences in the antitumor activity of the alkylating drugs in man. It must be emphasized immediately that there are obvious quantitative differences and that, because of ease of administration, lesser toxicity, and probable differences in distribution, certain of the compounds are far preferable to others in a particular clinical situation. The following quotation from Goodman and Gilman's *Textbook on Pharmacology* summarizes their advice on the choice of digitalis preparations. The aptness of it to the selection of alkylating agents in clinical cancer chemotherapy will be apparent.

"The physician has the choice of a large variety of preparations. The criteria of acceptability of a preparation include ease of administration, adequate intestinal absorption of therapeutically active amounts of orally administered drug, effective action, adequate margin of safety, stability, constant potency, reasonable cost, and rates of accumulation and elimination which permit one to treat at varying speeds and to maintain optimal effects over long periods."

Local Administration of the Alkylating Agents

Convincing evidence of the usefulness of intracavitary administration of the alkylating agents is presented, and the comparability of effectiveness to radioactive colloidal gold is demonstrated. It would appear that, because of the greater economy and safety of the chemotherapeutic method, the treatment of intractable neoplastic effusions by locally introduced drugs should supersede the use of radioactive isotopes. Nitrogen mustard or triethylene thiophosphoramide intrapleurally and Thio-TEPA intraperitoneally and intrapericardially are recommended.

The use of the alkylating drugs by intratumor injection is discussed. My conclusion is that the technique is of value only for cosmetic reasons that are relatively trivial in the management of disseminated neoplastic disease.

Alkylating Agents in Combination with Other Specific Therapeutic Measures

From animal experimentation it is suggested that nitrogen mustard in combination with urethan should receive clinical trial. This combination has not been studied. Reference is made in this monograph to combinations of nitrogen mustard with radiation in the treatment of bronchogenic carcinoma, and to nitrogen mustard and cortisone in the management of a variety of disseminated epithelial tumors. Although published reports favorable to these combinations have appeared in the literature, the evidence presented here fails to support them. Particularly convincing is the paper on the nitrogen mustard-cortisone combination, which shows clearly that the report in the literature was overly optimistic, and that subjective improvement too often must have been mistaken for objective benefit.

Chemoprophylaxis by the Alkylating Agents

This subject has become of considerable interest in surgical circles. It has been known for several years following the paper by H. C. Engell that tumor cells can be observed in the peripheral circulation of patients with cancer. In this country, emphasis has been placed on the phenomenon through the studies of W. H. Cole, which suggest that the exfoliation of tumor cells into the blood stream is increased during the surgical procedures of mobilization and removal of a cancer. This has led to Cole's experimental investigation of the effect of intraportal-vein, intraperitoneal, and intraperipheral-vein administration of triethylene thiophosphoramide on the "takes" in rats of Walker carcinoma 256 cells injected into the portal vein shortly before the drug. He found that a very significant reduction in the number of liver metastases could be achieved by this method of chemotherapy. In this monograph the toxicity of parenteral injection of nitrogen mustard in patients during surgery is presented, and some increase in postoperative bleeding is the major side effect noted. The experience in Hungary, Russia, and Japan on the combination of alkylating agents with surgery, in an attempt to improve the results of definitive cancer therapy in man, is presented. Apparently the multiplicity of variables has made it impossible to date to determine if the objective has been attained, although studies have been in progress for from three to five years.

It would appear to be important to determine experimentally whether chemoprophylaxis with the alkylating agents would be successful against a nonsusceptible tumor. Since the Walker rat carcinoma is unusually sensitive, whereas the human tumors in which the treatment is contemplated are, in the main, resistant to these drugs, the animal and human experiments are not analogous. The concept of chemoprophylaxis is attractive, and it is hoped that, through laboratory screening, agents will be found that will justify extensive clinical trial.

The Future of Alkylating Agents in Clinical Cancer Chemotherapy

Clinical studies are presented on the therapeutic usefulness of a number of nitrogen mustards in which the "carrier" moiety of the molecule is a sugar, an amino acid, or an antimalarial. These represent the vanguard of an almost limitless supply of nitrogen mustards. Are they all to be tried in man to determine whether their body distribution, tissue selectivity, or specific chemical reactivity has been modified so that they have a different spectrum of usefulness in cancer therapy than the agents now employed? Needless to say, and happily so, everyone will proceed as his heart, mind, and pocketbook dictate. To me, however, it would seem unwise, nay, foolhardy to attempt to screen these compounds in man. The rigid criteria for an objective and critical clinical evaluation of a drug against a variety of human cancers are such that each drug studied constitutes a major undertaking. Clinical observations should be reserved for those compounds that have been demonstrated in the laboratory either to have distinctive localization (anatomical or functional) or to have qualitative differences in their antitumor action from those alkylating agents now available. This obviously adds an additional major burden and responsibility to the laboratory, but considerations of precision of observation and patient safety would seem to dictate this course.

This monograph clearly demonstrates the value of these drugs in the medical management of human neoplastic disease. The permutations in the parent molecule have given to the clinic compounds that have significant quantitative differences, thereby facilitating variations in the use of the drugs. Further work in the synthesis of alkylating agents will undoubtedly continue, and it is hoped that clinical cancer chemotherapy will benefit through greater tumor specificity achieved by modification of tissue distribution.

FUTURE EXPERIMENTAL APPROACHES

By Alexander Haddow

Chester Beatty Research Institute, Royal Marsden Hospital, London, England

Any attempt to define future experimental approaches might well be regarded as presumptuous, yet we are constantly tempted to endeavor to do so. This monograph is timely, coming at the end of ten years' intensive investigation of these remarkable agents. It also may prove to be a milestone in the history of cancer research, and our successors will certainly look back upon it with interest.

A main purpose of the contributors to this publication has been to determine whether any of the alkylating agents have characteristic qualities or chemotherapeutic advantages that distinguish them from others. I am forced to conclude that there is no compelling evidence that this is so. On the other hand, the quantitative differences are of great interest, and even importance. If these agents were all simply of one species, we should expect them, as Galton pointed out, to be additive in their effects. This, however, is not the case, as shown by Elson's remarkable observation of the protective action of chlorambucil against the marrow-depressive action of Myleran. The situation appears to me to be similar to that existing between the alkylating agents and ionizing radiation. There is no questioning the validity of the radiomimetic comparison. In general, the alkylating agents mimic the action of ionizing radiations in an astonishing way. Equally, however, there are many points of difference in detail.

In discussing future experimental approaches, I shall divide my remarks into two parts; first, affecting the practical aspect of chemotherapy and, second, concerning more fundamental aspects.

I am fairly sure that the alkylating agents have no ultimate or permanent place in the chemotherapy of cancer. On the other hand, one cannot be entirely sure. In the last few years, we have seen for the first time instances of the cure of established animal tumors by purely chemical means. At the moment these are almost laboratory tricks. Conceivably, however, they could be reflected in the cure of human tumors by similar means a result that has not yet been achieved, but that must assuredly come. I therefore advocate, first, the continued synthesis and test of new alkylating agents, whether based upon sugars, amino acids, antimalarials, or other carriers and, second, the continued search for other alkylating side chains. The resources of cancer research are ample to permit this, without prejudice to any other approach. Mention has been made in these pages of the advantages to be gained from the successive use of different alkylating agents. I have been a little surprised, however, by the relative lack of reference to the advantages that may accrue from the simultaneous use of chemotherapeutic agents. In particular, I should plead for study of the combined effect of alkylating agents and urethan. Under experimental conditions these only summate, yet together they have given results more impressive than those obtained by any other combination. In man, the principle has been applied with success in the treatment of multiple myeloma. The various papers have reminded me of the dictum of William the Silent: "It is not necessary to hope, to begin; it is not necessary to succeed, to continue." We must go on, and results can still be obtained that are gratifying even if limited.

Having said all this, however, I remain convinced that the main and overriding importance of these agents lies in their fundamental interest and in the opportunities which they provide us to decipher the process of carcinogenesis and the process of mutagenesis, of which it is a special case. The alkylating agents are, as it were, reactive molecular spanners that we can throw into the cell machinery and that can send back information on its innermost secrets. These agents are relatively simple, often highly reactive, and have molecular features suggestive, if not indeed indicative, of likely modes of action. Ross has shown the relationship between chemical reactivity and biological action. The great question is, with what *significant* cellular receptors do the alkylating agents combine? Our earlier views as to a crosslinking mechanism were clearly unduly simple– crosslinking is probably sufficient and efficient, but not exclusive or necessary. However, the hypothesis was of the utmost fruitfulness, leading directly to the epoxides, imines, and dimesyl compounds, and its implications should still be pursued. Until recently there has been no suitable biological test system, but within the past few months Alexander has found evidence that such may be provided in the crosslinkage of the deoxyribonucleoprotein of herring sperm.

As to the nature of the receptor itself, there is much circumstantial evidence to suggest that the nucleic acid structure is at least a prominent candidate. The cytogenetic implications are immense, and more should be done to extend Revell's work as to the time of action of these compounds in interphase, the action on heterochromatin, and his conception that they may act, not by a process of random corrosion, but by a process more akin to the physiology of meiosis.

It is of tremendous interest that so many of the alkylating agents are powerfully mutagenic. Differences of opinion have been expressed as to the capacity of individual agents to effect specific or selective mutagenic effects. This question of differential mutagenesis is of the utmost importance to the geneticist and has wide implications far beyond the field of cancer. I cannot believe, however, that these considerations are of any immediate or primary importance for the cancer problem. What is important is that Fahmy has been able to suggest, first, a kind of radioequivalence in the mutagenic action of many of these compounds and, second, that they are capable of producing genetic deletion. It is a far cry from *Drosophila* to the mammalian cell, yet it well may be that these findings are highly relevant. Is malignant transformation due equally to somatic mutation by loss? In the last few years the evidence for this concept has been growing, even if it is not as yet complete. This view would have the widest implications. In many ways the rapidly growing embryonic cell is enzymatically deficient, although its growth is gradually brought under control by the orderly emergence of enzyme systems in ontogeny. Is it possible that the alkylating agents act as carcinogens by the deletion of enzyme systems normally responsible for growth control? If so, this view might eventually lead us to a new kind of chemotherapy based, not upon indiscriminate destruction, but upon the substitution of enzymatic control from without—by a kind of substitution therapy with which we are already familiar in diabetes and pernicious anemia.

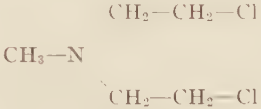
Of the very recent work that must be extended, I wish specially to refer to that of Roberts and Warwick. By an elegant use of radiographic chromatography, they have studied the action and fate of ethyl methanesulfonate and have demonstrated the appearance in the urine of various abnormal amino acids, and especially *S*-ethyl cysteine. There is no doubt as to the facts and, although we are of course uncertain as to their implications, they have forced us to reconsider the possibly vital role of interaction with SH groups and enzymes. Within the past few weeks tentative evidence has been obtained for growth-stimulating properties on the part of *S*-ethyl cysteine, and it is possible that these workers are on the verge of new developments.

To conclude: although the limited chemotherapeutic application of the alkylating agents is doubtless gratifying, I am convinced that their real importance is fundamental, through the light they may throw on the carcinogenic process and on all that this may mean.

APPENDIX

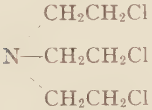
Structural Formulas of Principal Compounds Used Clinically

Compiled by David A. Karnofsky



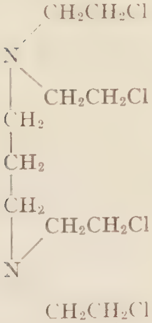
$$\mathrm{CH_3{-}N}\begin{matrix}\diagup\mathrm{CH_2{-}CH_2{-}Cl}\\ \diagdown\mathrm{CH_2{-}CH_2{-}Cl}\end{matrix}$$

Methyl-bis(β-chloroethyl)amine
(HN2, Mustargen)

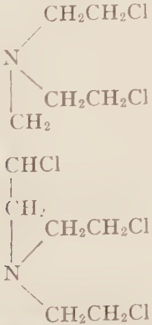


$$\mathrm{N}\begin{matrix}\diagup\mathrm{CH_2CH_2Cl}\\ {-}\mathrm{CH_2CH_2Cl}\\ \diagdown\mathrm{CH_2CH_2Cl}\end{matrix}$$

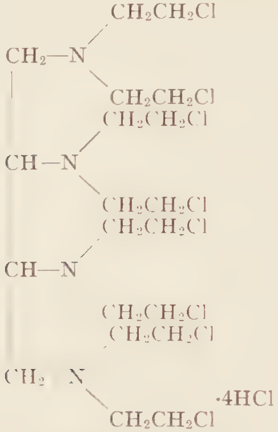
Tris(β-chloroethyl)amine
(HN3)



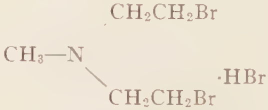
1,3-Diaminotetrakis(*N*,*N*,*N′*,*N′*-β-chloroethyl)propane
(SK 136)



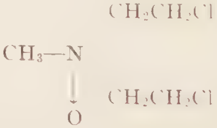
1,3-Diamino-β-chlorotetrakis(*N*,*N*,*N′*,*N′*-β-chloroethyl)propane
(SK 137)



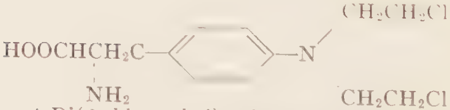
1,2,3,4-Tetrakis[(bis-β-chloroethyl)amino]butane tetrahydrochloride
(SK 596)



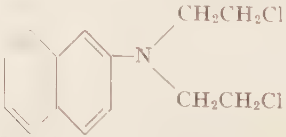
Methyl-bis(β-bromoethyl)amine hydrobromide
(SK 437)



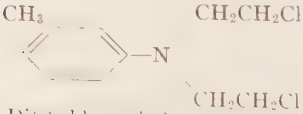
Methyl-bis(β-chloroethyl)amine *N*-oxide
(Nitromin, Mitomen, Nitro-Lost)



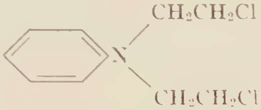
p-Di(β-chloroethyl)aminophenylalanine
(racemic form, sarcolysin; L-isomer, CB 3025, Melphalan)



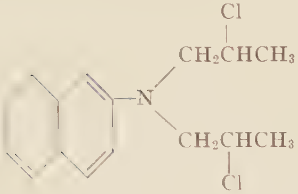
β-Naphthyl-di(β-chloroethyl)amine
(R 48, CB 1048)



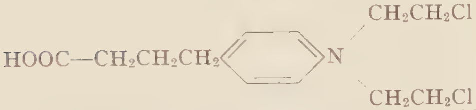
Di(β chloroethyl) *m*-toluidine
(R 50)



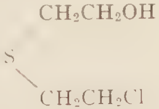
Di(β-chloroethyl)aniline
(R 74)



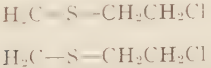
β-Naphthyl-di(β-chloropropyl)amine
(R 151)



p-*N*,*N*-Di(β-chloroethyl)aminophenylbutyric acid
(CB 1348, chlorambucil, Leukeran)

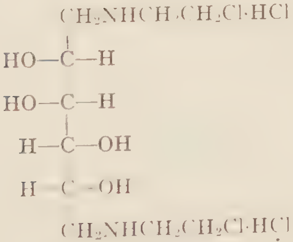


2-Chloro-2′-hydroxydiethyl sulfide
(HSM, hemisulfur mustard)



$$\begin{array}{l} H_2C-S-CH_2CH_2Cl \\ H_2C-S-CH_2CH_2Cl \end{array}$$

1,2-Bis(β-chloroethylthio)ethane
(HSM-1)

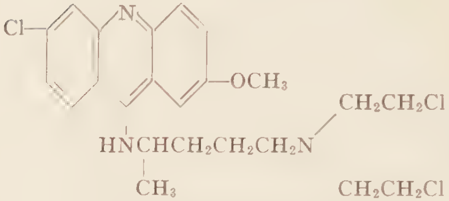


$$\begin{array}{c} CH_2NHCH_2CH_2Cl \cdot HCl \\ | \\ HO-C-H \\ | \\ HO-C-H \\ | \\ H-C-OH \\ | \\ H-C-OH \\ | \\ CH_2NHCH_2CH_2Cl \cdot HCl \end{array}$$

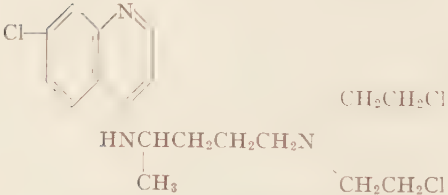
1,6-Bis(2-chloroethylamino)-1,6-deoxy-D-mannitol dihydrochloride
(BCM, Degranol)

$CH_3SO_2-O-CH_2CH_2Cl$

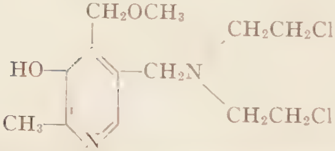
β-chloroethylmethane sulfonate
(CB 1506)



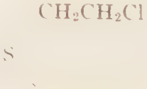
Quinacrine nitrogen mustard



Chloroquine nitrogen mustard

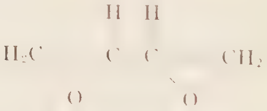


3-Bis(β-chloroethyl)aminomethyl-4-methoxymethyl-5-hydroxy-6-methylpyridine
(Methoxypyridoxine nitrogen mustard, SK 1424)

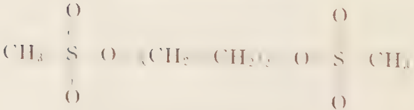


$$S\begin{array}{l}CH_2CH_2Cl\\CH_2CH_2Cl\end{array}$$

Bis(β-chloroethyl) sulfide
(Sulfur mustard, H)

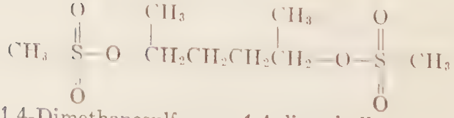


Butadiene dioxide



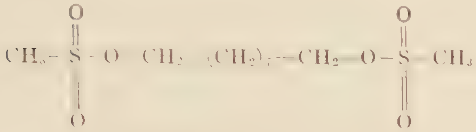
$$CH_3-\overset{O}{\underset{O}{S}}-O-(CH_2-CH_2)_2-O-\overset{O}{\underset{O}{S}}-CH_3$$

1,4-Dimethanesulfonoxybutane
(GT 41, busulfan, Myleran)



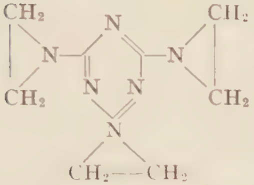
$$CH_3-\overset{O}{\underset{O}{S}}-O-\overset{CH_3}{CH_2}CH_2CH_2\overset{CH_3}{CH_2}-O-\overset{O}{\underset{O}{S}}-CH_3$$

1,4-Dimethanesulfonoxy-1,4-dimethylbutane
(CB 2348, dimethyl Myleran)

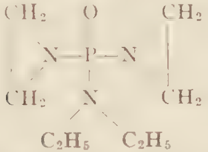


$$CH_3-\overset{O}{\underset{O}{\overset{\|}{\underset{\|}{S}}}}-O-CH_2-(CH_2)_7-CH_2-O-\overset{O}{\underset{O}{\overset{\|}{\underset{\|}{S}}}}-CH_3$$

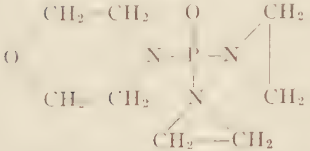
1,9-Dimethanesulfonoxynonane
(Nonane)



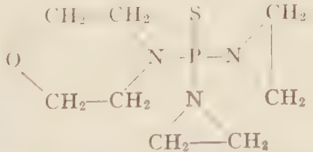
Triethylene melamine
(2,4,6-Triethylenimino-*s*-triazine, TEM)



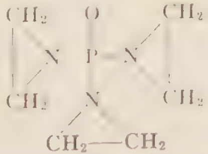
Diethylene phosphoramide
(DEPA)



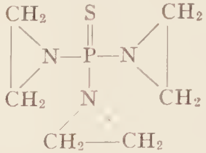
N-3-Oxapentamethylene-*N*′,*N*″-diethylene phosphoramide
(ODEPA)



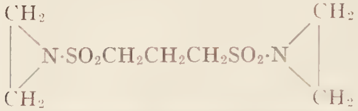
N-3-Oxapentamethylene-*N*′,*N*″-diethylene thiophosphoramide
(OPSPA)



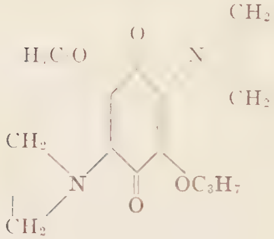
Triethylene phosphoramide
(TEPA)



Triethylene thiophosphoramide
(Thio-TEPA, TSPA)



1,3-Bis(ethyleniminosulfonyl)propane
(BEP)



2,5-Diethylenimino-3,6-diproproxy-1,4-benzoquinone
(E 39)

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